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TITLE: Prevention and Treatment of Breast Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project is to test the hypothesis that AFPep is safe and effective for prevention and treatment of breast cancer. Aim 1 is to document the preventive efficacy and safety of AFPep. The data reported herein allow us to conclude that AFPep prevents estrogen-induced breast cancer in ACI rats (just as it prevented carcinogen-induced breast cancer in Sprague Dawley rats). From the ACI rat model, together with data in primates (primate work not supported by CDMRP) and data from dogs, we conclude that AFPep is extraordinarily safe. Aim 2 is to measure and maintain blood levels of AFPep at effective doses in mice, dogs, and non-human primates. Assessing pharmacokinetic data in mice and primates, we conclude that an efficacious blood level of AFPep is approximately 1 µg/mL, and this blood level can easily be achieved in higher mammals with no evidence of toxicity. Although there is important work to be done in Years 2 and 3 of this grant, we conclude from the data of Year 1 that AFPep is safe and effective for the prevention and treatment of breast cancer, and should proceed to clinical trials for the prevention of breast cancer as soon as possible.

15. SUBJECT TERMS

Breast Cancer; Prevention: Therapy: Pharmacokinetics; Therapeutic Index; Safety

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1. INTRODUCTION:

This proposal offers solutions for two overarching challenges in that it will demonstrate: *a.*) primary chemoprevention of breast cancer and *b.*) treatment regimens for ER+ breast cancer that are safe. The Breakthrough that is needed to realize the CDMRP's Vision "to eliminate breast cancer" is a chemopreventive agent that is as safe as it is effective. The subject of this project is just such a molecule: AFPep is a first-in-class, well tolerated, growth regulatory synthetic peptide that stops development and growth of breast cancer in rodents. AFPep is a small molecule mimic of the active site of a naturally occurring protein of pregnancy (α-fetoprotein) which is largely responsible for the lifetime reduction in risk of breast cancer that occurs as a result of pregnancy. The scope of this translational project entails essential work that will move AFPep beyond discovery and position it to enter clinical trials. The purpose of the research is to test **the hypothesis that AFPep is safe and effective for prevention and treatment of breast cancer.** There are two specific aims being studied simultaneously: 1) Use an innovative cancer prevention model in rats to document the preventive efficacy and safety of AFPep. We show herein that AFPep can offer primary prevention, and that it is extraordinarily well tolerated. 2) Use a veterinary clinical trial in dogs to document efficacy of AFPep against spontaneous heterogeneous mammary cancer. We show herein that we can measure and maintain blood levels of AFPep at effective doses in mice, dogs, and non-human primates.

In addition to the effort supported by this CDMRP grant, we have data that were supported by funding from the Albany Medical College (AMC). Some parts of our study reported below were supported entirely by intramural funds, ***not DOD-BCRP funds,*** *but certainly speak directly to our hypothesis. Assurances are given that all studies were in accord with AMC IACUC oversight. A unique and time-sensitive opportunity arose at our medical center in which five monkeys (Macaca mulatta, males 11 years in age and 8 kg in weight) became available to us between December 2015 and June 2016 for studies of safety and pharmacokinetics of AFPep. These data are reported below, and demarcated by being in Arial italic font.*

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Breast Cancer; Prevention; Therapy; Pharmacokinetics; Safety; Therapeutic Index; Pharmacodynamics

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

The goals of the project are outlined in the approved Statement of Work. For tasks that require elaboration (as indicated in the Comment column), we provide detailed information in subsequent paragraphs.

This is the approved SOW for Specific Aim 1:

Specific Aim 1 Demonstrate that AFPep will interdict breast cancer at any stage of progression	**Proposed Timeline**	**Progress**	**Comment**
Major Task 1 Start-up tasks	Months		
Subtask 1 IACUC approval	-3	Complete	Initial Comments
Subtask 2 Pre-engage CRO for drug synthesis	-2	Complete	
Subtask 3 Pre-engage animal supplier for rat acquisition	-3	Complete	
Subtask 4 Hire technician	1	Complete	
Subtask 5 Test quality of drug	2	Ongoing	1
Major Task 2 Determine dose-response curve for prevention in ACI rats			
Subtask 1 Acquire rats, implant with estrogen pellets	2	Complete	2
Subtask 2 Monitor rats for tumors	3 - 13	Ongoing	2
Subtask 3 Necropsy of animals; pathology analysis	12-13	Started	3
Subtask 4 Ongoing quality analyses of commercial AFPep	1 - 36	Ongoing	1
Milestone(s) Achieved: Determination of dose-response curve of AFPep for prevention of cancer; determination of safety of AFPep	13	Ongoing	3
Major Task 3 Demonstrate interdiction of cancer progression			
Subtask 1 Acquire animals, implant with estrogen pellets	13	Year 2	
Subtask 2 Monitor animals for tumors	14 - 24	Year 2	
Subtask 3 Necropsy of animals; pathology analysis	23-24	Year 2	
Subtask 4 Preparation of manuscript for publication	23-25	Year 2	
Milestone(s) Achieved: Interdiction of progression of cancer	2 years	Year 2	
Major Task 4 Determine minimal duration of treatment sufficient to produce life-long prevention			
Subtask 1 Acquire animals, implant with estrogen pellets	25	Year 3	
Subtask 2 Monitor animals for tumors	25-36	Year 3	
Subtask 3 Necropsy of animals; pathology analysis	35-36	Year 3	
Subtask 4 Preparation of manuscript for publication	35-36	Year 3	
Milestone(s) Achieved: Determine optimal schedule for AFPep use	Year 3	Year 3	

Initial Comments: All of the start-up activities were completed. Arrangements for CDMRP IACUC processes and CDMRP budgeting processes took many weeks, and were handled sequentially instead of concomitantly (which would have been possible and would have saved several weeks). Arrangements at Albany Medical College for hiring a technician (see below), animal care and use procedures, and arranging with animal and drug suppliers went well and in a timely fashion.

What was accomplished under these goals?

Comment 1. Relating to *Specific Aim 1*, Major Task 1, *Subtask 5:* "Test quality of drug," and to *Specific Aim 1*, Major Task 2, *Subtask 4:* "Ongoing quality analyses of commercial AFPep"

AFPep was purchased from AmbioPharm, Inc. and subjected to a number of quality control procedures. Because the prevention studies (described below) span several months, it is essential to demonstrate that the drug: a.) remains consistently active over the duration of the study, b.) can be synthesized reproducibly, and c.) remains unchanged in blood so as to be quantifiable. All of these considerations were achieved.

a.) Shown in Figures 1 and 2 is that AFPep retains its biological activity for months, whether stored at – 80 C or at -20 C. It should be noted that the duration of the prevention study reported below is 28-30 weeks.

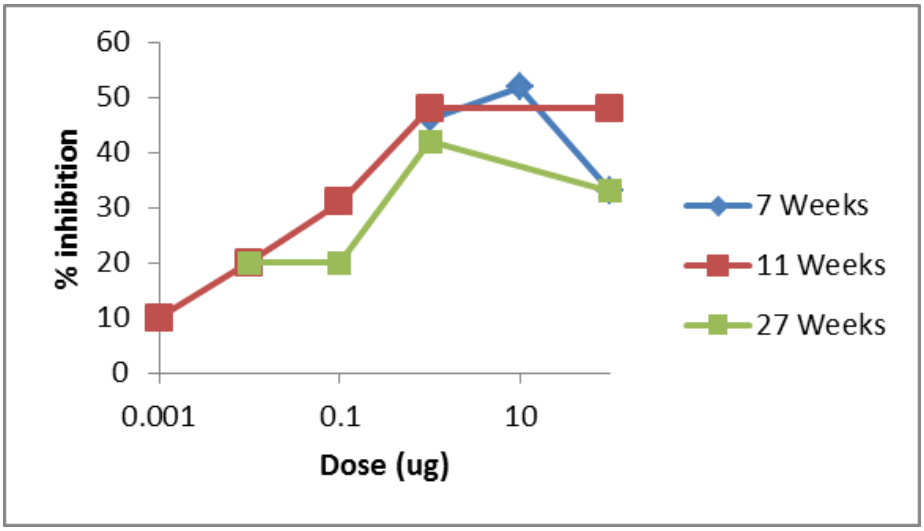


Figure 1. AFPep retains biological activity after several weeks in storage at – 80 C. AFPep was received from the commercial supplier and stored at -80C. It was subjected to bioassay (inhibition of estrogen-stimulated uterine growth in an immature mouse) to assess potency. The y-axis reports inhibition of estrogen-stimulated weight gain of the uterus 24 hours after exposure to estrogen and AFPep. Assays were conducted at the storage durations indicated in the figure. There is no loss of activity as a function of time in storage.

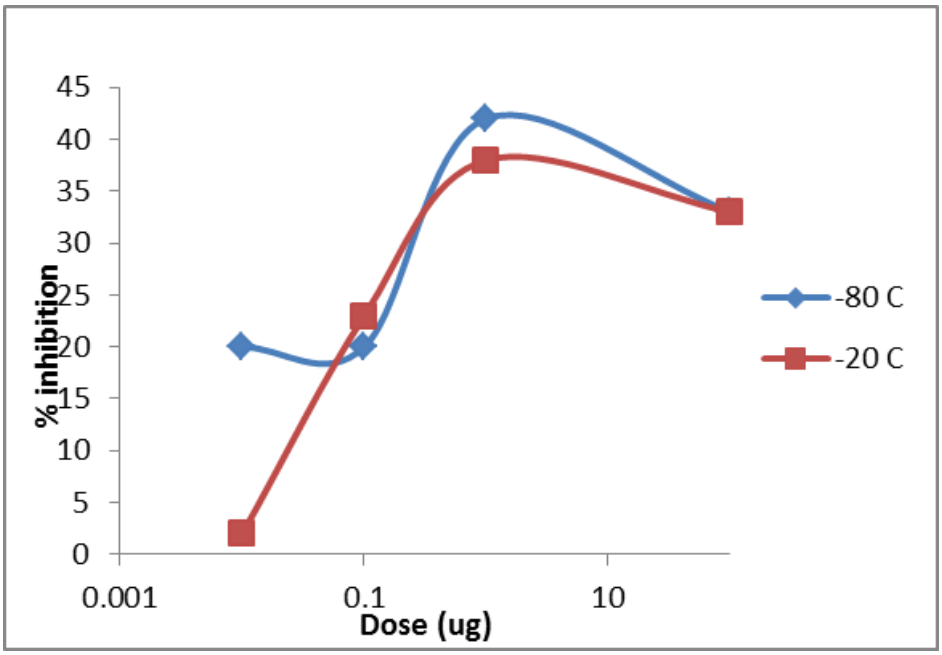


Figure 2. AFPep retains biological activity after several weeks in storage at – 80 C or at -20C. AFPep was received from the commercial supplier and stored at -80C. Another portion of the drug was retained at AmbioPharm, where they stored it at -20 C. Samples were subjected to bioassay (inhibition of estrogen-stimulated uterine growth in an immature mouse) to assess potency after 20 weeks. The y-axis reports inhibition of estrogen-stimulated weight gain of the uterus 24 hours after exposure to estrogen and AFPep. There is no difference in activity as a function of storage temperature.

b.) To demonstrate that the commercial supplier can prepare AFPep reproducibly, Figure 3 compares activity in the mouse uterine growth inhibition assay of two completely different syntheses of AFPep done at AmbioPharm. Quality control data from the supplier showed no differences between synthetic batches when compared by high pressure liquid chromatography, mass spectroscopy analysis, and other routine analyses for synthetic peptides (data not shown).

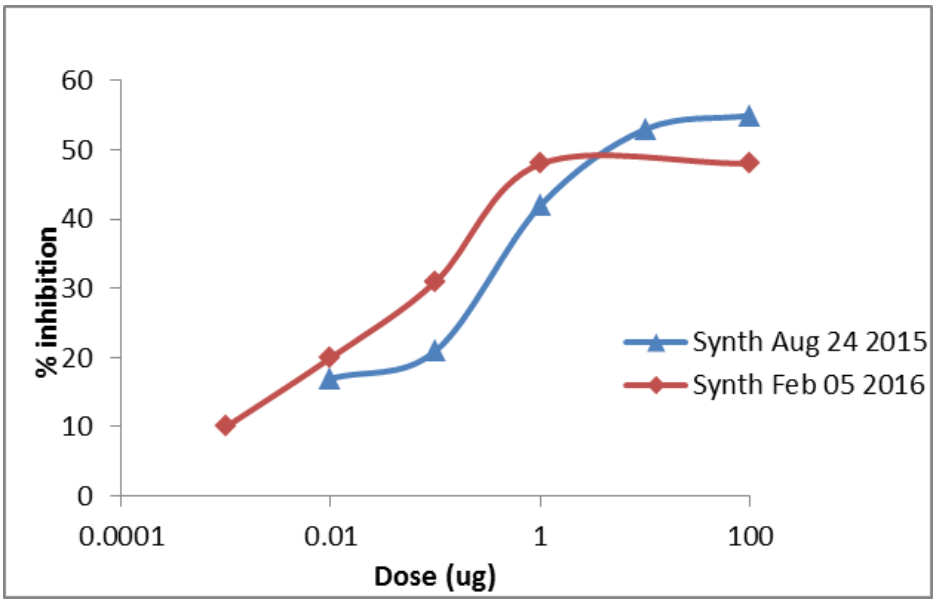


Figure 3. The biological activity of AFPep does not differ among repeated synthetic preparation of the drug. AFPep was synthesized by a commercial supplier; AmbioPharm has been consistent in providing high quality AFPep, and is capable of making GMP-quality drug when necessary. Here, two different synthetic preparations are compared in the mouse uterine growth inhibition assay; the activity is indistinguishable between preps.

c.) In addition to storage (shelf life) stability, it is important to demonstrate the stability of AFPep in serum to be sure that pharmacokinetic samples can be processed and analyzed without concern for drug degradation. For that demonstration, we compared samples that had been stored in saline or serum, and for various durations, as shown in Figure 4.

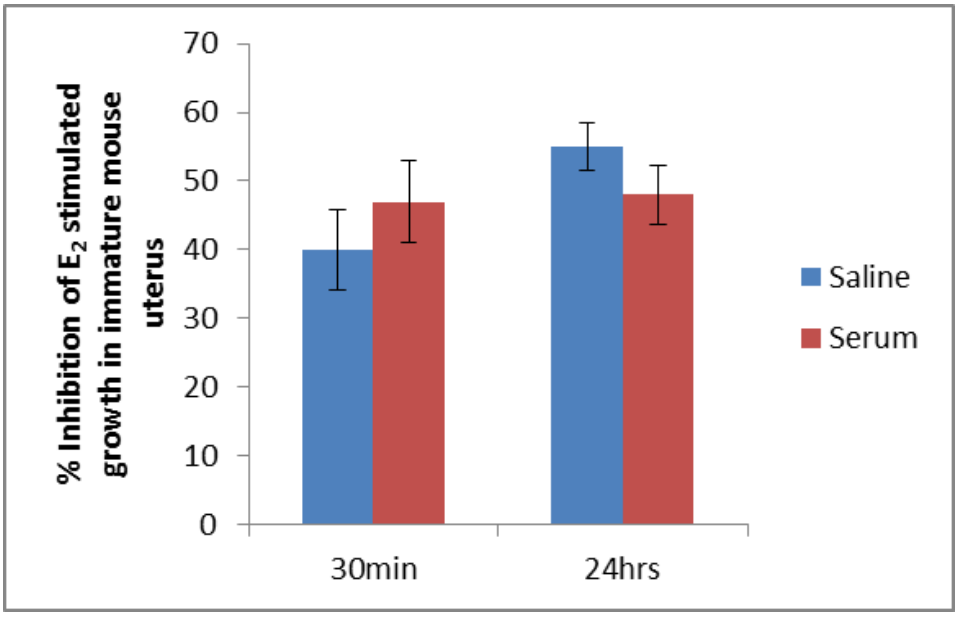


Figure 4. AFPep is stable when stored in either saline or serum. AFPep was dissolved in the indicated solvent and allowed to incubate for the indicated times at room temperature. Subsequently, the drug was used in the mouse uterine growth inhibition assay to assess biological activity. There are no significant differences in biological activity following solubilization of AFPep in either saline or mouse serum. In addition, even lengthy incubation at room temperature is not detrimental to AFPep.

As another assessment of stability, and in preparation for pharmacokinetic analyses (see **Comment 4** below), we used liquid chromatography coupled with tandem mass spectrometry. This LC-MS/MS method for quantification of AFPep in mouse plasma was done in conjunction with Dr. Qishan Lin at the Proteomics Institute at the University at Albany (on a fee-for-service basis). The developed method was validated in terms of accuracy, precision, selectivity, sensitivity, stability and reproducibility. The developed method was found to be accurate and precise with LLOQ and LLOD of 0.2 ng/ml and 1 ng/ml, respectively; method selectivity was confirmed by the absence of any matrix interference with the analytic peak. The constructed calibration curve was linear in the range of 1-2,000 ng/ml, with a regression coefficient of 0.998. As shown in Figure 5, AFPep was found to be stable in mouse plasma at room temperature for 24 hrs. The established method provides rapid, sensitive, rugged, and robust LC-MS/MS analysis for the quantitative determination of AFPep in biological matrices.

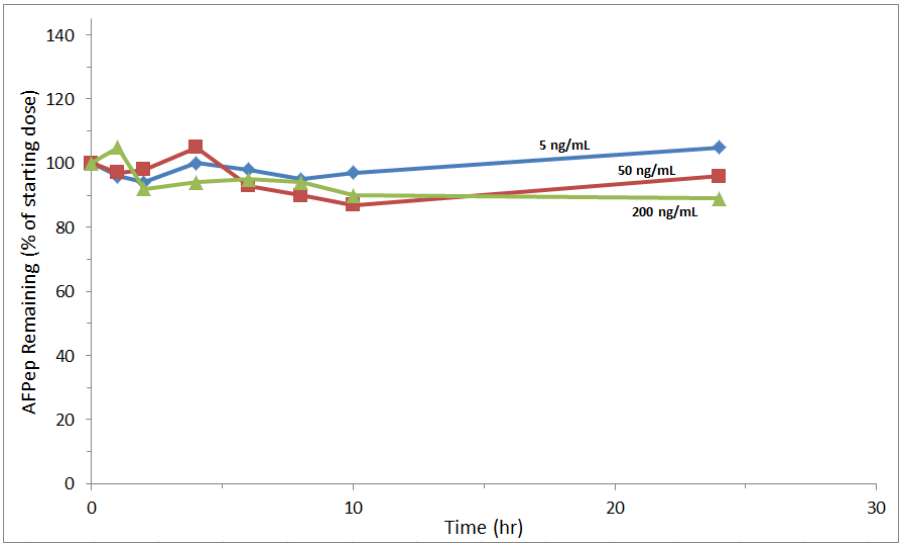


Figure 5. AFPep is stable during incubation at room temperature in mouse plasma, as shown by LC/MS-MS. AFPep was incubated at 5 µg/ml, 50 µg/ml, or 200 µg/ml at room temperature for 24 hours, then analyzed as described in the text. There is no loss of AFPep and no change in its structure.

Comment 2 Relating to *Specific Aim 1*, Major Task 2, *Subtasks 1 and 2* "Prevention of Mammary Cancer in ACI rats."

Subtask 1: Acquiring ACI rats.

In our previous publications, we showed that AFPep prevents mammary cancer in Sprague Dawley (SD) rats exposed to a harsh carcinogen (methyl nitroso urea, MNU). The purpose of the present project is to show that AFPep will prevent mammary cancer in a rat model that is much more similar to human breast cancer. This model uses ACI rats, in whom cancer reproducibly develops in response to estrogen maintained in the high physiological range. There is only one supplier of ACI rats (formerly known as Harlan Sprague Dawley, now known as Envigo, Inc.). The supplier is not willing to maintain a colony large enough to supply hundreds of animals at a time, but they will supply approximately 20 animals at a time. Therefore, in order to implement the study shown in Table 1 which requires 170 animals, we received 10% of the sample size in each shipment and started the experiment 10 times. That is to say, we received 17 animals at a time, spaced two weeks apart. The implications of this experimental design are that the experiment lasts much longer than the 200 days anticipated. Thus, the study is not completed as of the time of writing of this Progress Report. Some of the statistics are not finalized for the prevention data, though the trend is clear. The safety data are sufficient to achieve statistical significant results. Both safety and efficacy data are reported below.

It should be noted that there are two major changes between these studies and our earlier demonstration of prevention of breast cancer, namely the method of inducing cancer/strain of rat, and the duration of treatment with AFPep. Each has important consequences.

- The MNU/SD model can complete a study within 100 days, but the estrogen/ACI model requires at least twice as long. Although this model is much more expensive and time consuming, it is much more similar to breast cancer in women than is the carcinogen-induced models. Thus effective prevention in this model is very exciting. In addition, because the study takes so long, it is ideal for assessing safety simultaneously with efficacy. The data below show that extensive treatment (duration and dose) with AFPep is very well-tolerated. AFPep is non-toxic, and led to no discernable side effect.
- For the MNU/SD model, we treated rats for only 23 days, a duration chosen to mimic pregnancy. Because AFPep has its origins in mimicking α-fetoprotein (a protein produced by the liver of the fetus), we had wanted to demonstrate that it was working through the AFP mechanism. That study demonstrated very good prevention, but it was not known if even better prevention could be obtained if treatment had been life-long. Thus, for this estrogen/ACI study, we treated rats continuously (5 days on/2 days off) for 30 weeks. In these estrogen/ACI studies, preventive efficacy is not superior to that in the shorter duration MNU/SD model. Therefore, the data show that duration of treatment does NOT need to be life-long, that brief treatment (probably similar to duration of pregnancy) may be optimal. This bodes well for women who might want to use AFPep to prevent breast cancer. (N.B.: Our Year 3 prevention trial was designed to test duration of administration.)

Considerations of Experimental Design: This experimental design allows assessment of both safety and efficacy. Group 1 is a negative control: animals that do not receive estrogen should not get cancer, and none did. Group 2 is a positive control: animals that get only estrogen should develop mammary cancer, and most did. Groups 3 through 6 are a dose response analysis of AFPep; results are discussed below. Group 7 is high dose AFPep, no estrogen. This group should not get mammary cancer (none did), and serves to demonstrate any side effects or toxicity of AFPep (no side effects, no toxicities were observed).

Table 1. **Prevention Trial A: AFPep Dose-Response Curve** ***Does AFPep prevent estrogen-induced breast cancer?***

Group No.	No. of Animals	Estrogen	AFPep ug/kg/day	*ug/rat/day*	Duration of AFPep
1	10	-			-
2	30	+			-
3	30	+	50	*10*	Continuous 200 days
4	30	+	500	*100*	Continuous 200 days
5	30	+	2500	*500*	Continuous 200 days
6	30	+	5000	*1000*	Continuous 200 days
7	10	-	5000	*1000*	Continuous 200 days
	170	***Total number of animals***			

Subtask 2: Monitor for tumors. Prevention of breast cancer in ACI rats.

Our published work showed that AFPep powerfully prevented carcinogen-induced breast cancer in Sprague Dawley rats (MNU/SD model). The purposes of Prevention Trial A are to show that AFPep prevents estrogen-induced breast cancer in ACI rats (estrogen/ACI model), to determine the optimal dose of drug, and to assess safety and side effects. We have accomplished all of these purposes, although this study is not yet complete (due to the staggered start described above). Consequently, we do not report detailed statistics, but these will become available after completion of the trial.

AFPep prevents estrogen-induced breast cancer in ACI rats. As shown in Figure 6, incidence of mammary tumors was decreased by 50% in estrogen-exposed rats treated with 100 µg of AFPep compared to estrogen-exposed, saline-treated rats. The dose-response effect is similar to what was observed earlier with Sprague

Dawley (data not shown) rats in that 100 µg (only 100 nanomoles) provides optimal protection. Higher doses were not necessary, and in fact the highest dose led to less protection (see comments below). This bodes very well for women who want protection from breast cancer but who do not wish to be over-medicated.

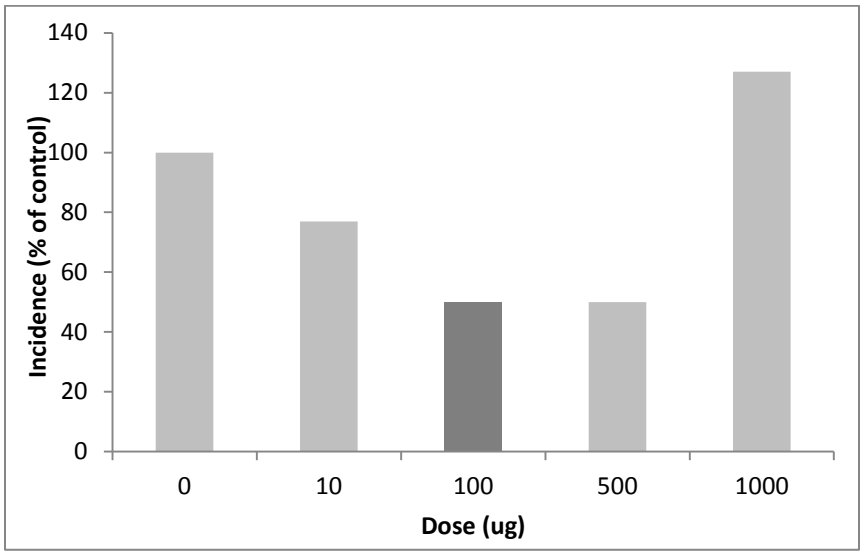


Figure 6. AFPep reduces the incidence of estrogen-induced breast cancer. ACI rats were implanted with an estrogen-containing Silastic tube, then treated s.c. with the indicated dose of AFPep continuously (5 days on/2 days off) for 30 weeks. Incidence (defined as number of animals that have one or more palpable tumors) is shown here as a % of the No AFPep group. There were 30 animals per group to ensure a power of detecting a difference between groups of 87%. AFPep at 100 µg/rat decreased incidence by 50 %.

Figure 7 shows that AFPep increased the latency, the time from onset of estrogen treatment to appearance of tumors. Treatment of animals with 100 µg of AFPep led to a 25 % increase in latency (from 15 to 19 weeks) compared to estrogen-exposed, untreated animals. This increase is actually better than the latency enhancement in the MNU/SD model (data not shown). Here again higher doses of AFPep were not more effective.

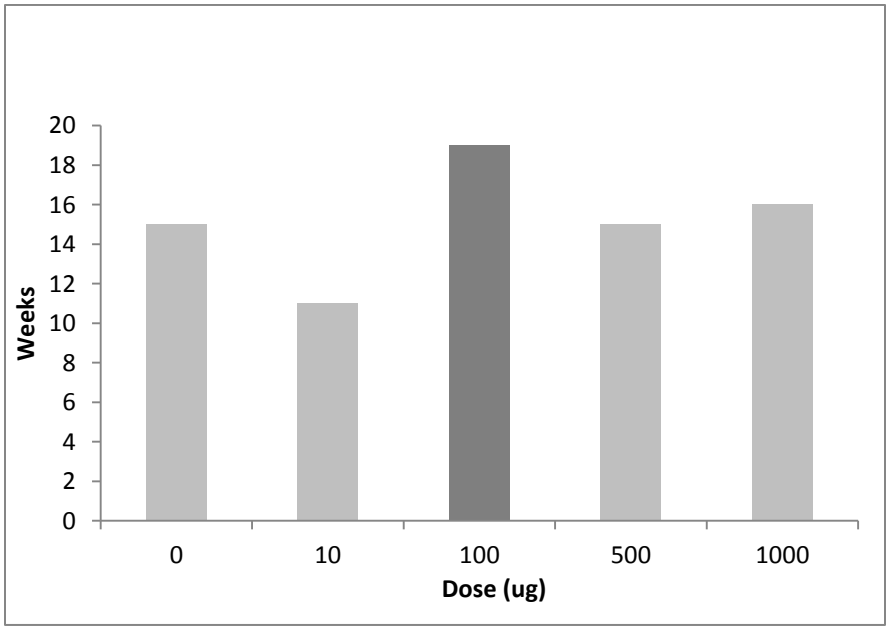


Figure 7. AFPep delays onset of breast cancer. Methodology as in Figure 6; latency is defined as the number of weeks before tumors appear in any animal within the group. N = 30 animals per dose. The optimum dose of AFPep is 100 µg/animal.

Thus we have established that AFPep prevents estrogen-induced breast cancer and have established the optimal dose to be 100 µg/animal. This will allow us to proceed to the Year 2 and Year 3 prevention trials.

The observation that higher doses of AFPep are unnecessary is interesting and deserves comment. In earlier publications, we postulated a model featuring two receptors for AFPep (and, by extension, for the parent protein AFP, which was also postulated previously (by others) to have a two-receptor mechanism). We articulated the concept that binding of AFPep to the higher affinity receptor produced the desired result of breast cancer inhibition, and that binding of AFPep to the lower affinity receptor led to an undesirable effect, namely a reversal of the inhibition. We have observations from a number of different assays, in mice, rats, and human cell culture in support of the two-receptor model and suggesting that higher doses of AFPep are unhelpful. The current study offers the most substantial confirmation of this model to date, in that higher doses of AFPep were not more effective at prevention of breast cancer. Our data also suggest that continuous treatment even at low doses may not be helpful: continuous treatment for 200 days led to no enhancement of preventive efficacy compared to that in the MNU/SD model in which treatment was for a short duration. This is a very important observation, and a profound benefit of having opted for this experimental design. We note that if we had not chosen to do life-long treatment in this trial, the data would have compelled an additional trial to resolve the duration of treatment question. As it is, we are now in a very strong position to move forward with Year 2 Prevention Trial B (which asks the question *Is it ever too late to prevent breast cancer?*) and with Year 3 Prevention Trial C (which asks *How brief a treatment with AFPep is sufficient to provide maximal benefit*?). However, the Year 3 trial is now of paramount importance, and we will do it in Year 2.

We have another intriguing observation that substantiates the conclusion that low dose/short duration treatment may be optimal, although we do not have statistically significant data as of yet. Because the animal supplier always sent us one more animal than we ordered (presumably because these animals sometimes die during shipping), we had three extra animals that were treated briefly with the lowest dose (10 µg) of AFPep but were subsequently withdrawn from the study. Rather than sacrificing these animals, they were maintained on saline and estrogen, but got no further AFPep. As of this time, none of those animals have developed tumors, whereas untreated animals begin developing tumors by 15 weeks after implanting estrogen. Based on Latency, this suggests, *without statistical power*, that short duration treatment with low dose AFPep may be especially effective for prevention of mammary cancer. Based on Incidence, this small study may achieve statistical significance at the end of the experiment, especially if these three animals never develop a tumor, but the results are not yet statistically significantly different from either the 100 µg dose group or from the 0 µg dose group. We comment on it here in order to highlight the importance of the Year 3 Prevention Trial which now seems paramount (compared to the Year 2 Prevention Trial). We intend to do the Year 3 trial next so that we can know the optimal duration of treatment before trying to interdict cancer after onset.

Table 2. Observations suggesting that short duration treatment with AFPep is sufficient for prevention
As of 8 AUG 2016

Ear Tag	Duration of AFPep Treatment	Weeks after implant of E2	Number of Tumors
321	8.5 weeks	25	0
374	1.5 weeks	18	0
400	4.5 weeks	21	0

We conclude that AFPep prevents estrogen-induced breast cancer in ACI rats (just as it prevented carcinogen-induced breast cancer in Sprague Dawley rats), and should proceed to clinical trials for the prevention of breast cancer as soon as possible.

Comment 3 Relating to *Specific Aim 1*, Major Task 2, *Subtask 3* "Pathology Analyses" and "Milestones"

Subtask 3: Safety study: Necropsy of animals; pathology analysis. Milestone: Determination of safety

Animal weights and organ weights can serve as indicators of toxicity in animal being treated with potential drugs. ACI rats arrive from the supplier at approximately 90 – 110 grams in body weight, and are maintained in the AMC Animal Resource Facility until they achieve a weight above 110 g, at which time a Silastic tubing implant (containing nothing or containing estrogen) is implanted subcutaneously. Animals then begin treatment with AFPep and weight is monitored weekly. Shown in Figure 8 are the average body weights of groups of animals (groups defined as in Table 1). It is apparent that estrogen-exposed animals develop a heavier weight than do animals not exposed to estrogen. It is also apparent that AFPep does not affect body weight, whether the animal was exposed to estrogen or not.

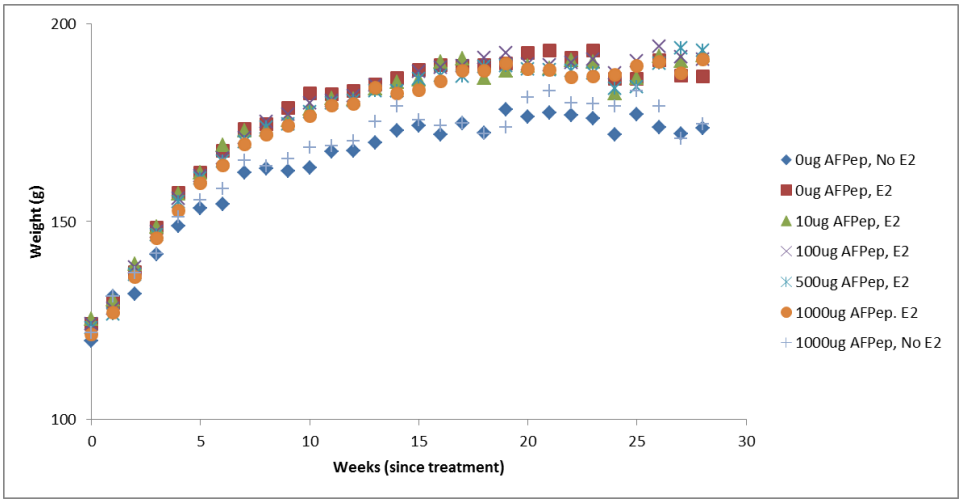


Figure 8. AFPep does not affect animal body weight. ACI rats increase in weight up to approximately 200 grams over their lifetime. Animals were implanted with empty Silastic tubing implants or with implants containing estrogen when they achieved a weight of > 110 g; time of implantation is defined as week 0 of treatment. Animals were weighed at weekly intervals. Shown here are average weights of animals within a Group (as defined in Table 1). Animals that were exposed to estrogen developed a greater body weight compared to those that did not receive estrogen ($p < 0.02$). AFPep did not affect body weight, either in the presence or absence of estrogen ($p < 0.88$).

To analyze more closely the statistics of weight gain, Figure 9 shows average weights within a group at three intervals throughout the study. All animals begin at the same weight, but by Week 15 of treatment, the estrogen effect is detectable. At the end of the study, the effect of estrogen is statistically significant. There is no effect of AFPep on average animal weights.

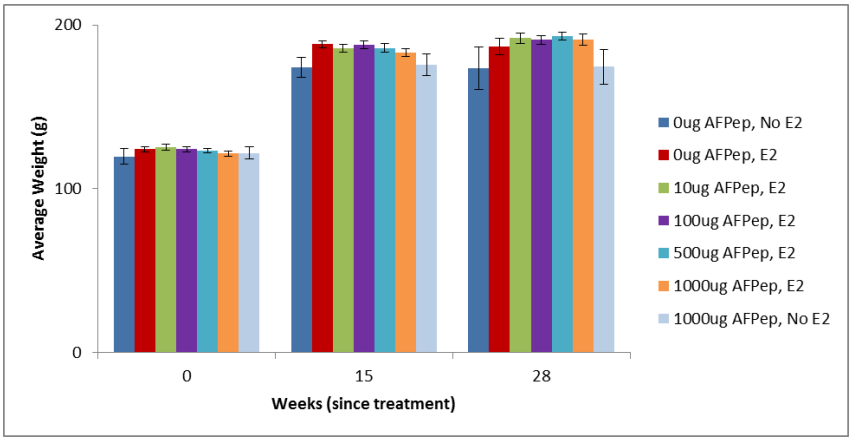


Figure 9. AFPep does not affect animal body weight. There are no statistically significant differences between Groups at the onset of treatment. At 15 weeks of treatment, estrogen treated animals are heavier than animals not exposed to estrogen ($p < 0.02$). n = 30 animals in Groups 2 through 6; n = 10 animals in Groups 1 and 7.

Shown above are average weights for groups of animals (groups defined as in Table 1). To assess whether a few individual animals may have been affected by AFPep, Figure 10 shows, in Box and Whisker format, the individual animal weights at 14 weeks of treatment. There is no statistical difference between AFPep-treated and non-treated animals in groups without estrogen (i.e., Group 7 vs. Group 1), indicating AFPep does not affect the weights of even a few animals. There is no statistical difference among estrogen-exposed Groups that were treated with various doses of AFPep (i.e., Groups 3 through 6), again indicating that AFPep does not affect the weight of even a few animals.

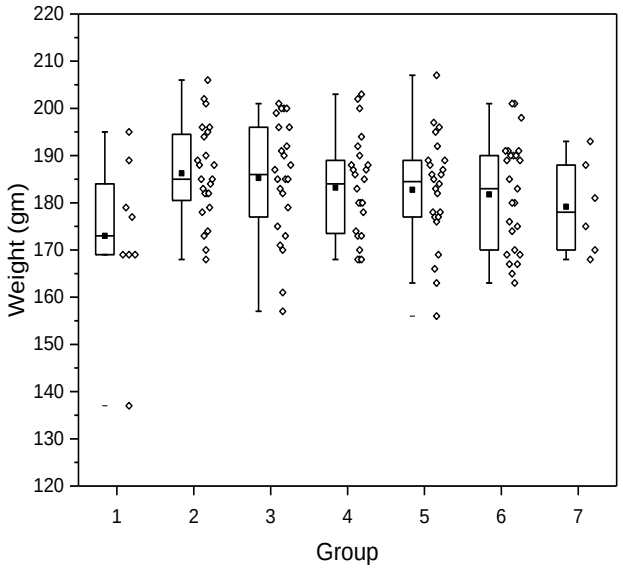


Figure 10. Treatment of ACI rats with AFPep for 14 weeks does not introduce weight differences. This box-and-whisker format portrays individual rat weights at a single time point within each Group (shown as individual data points), as well as the high weight and low weight (whiskers), and 25th to 75th percentile (box). Groups are defined in Table 1. There is an outlier animal in Group 1 (No estrogen, No AFPep); that animal was always healthy, but simply smaller than littermates.

As a further assessment of potential toxicity, organ weights were examined at autopsy. Animals were sacrificed when they reached 28 weeks after implantation of estrogen. Figures 11 (organ weight) and 12 (organ weight/body weight ratio) show that there was no effect of estrogen or AFPep on organ weights (or normalized organ weights) for lung, kidney, or heart. For liver, the difference due to estrogen is small and not yet statistically significant (not all Groups have been sacrificed). There is no effect attributable to AFPep.

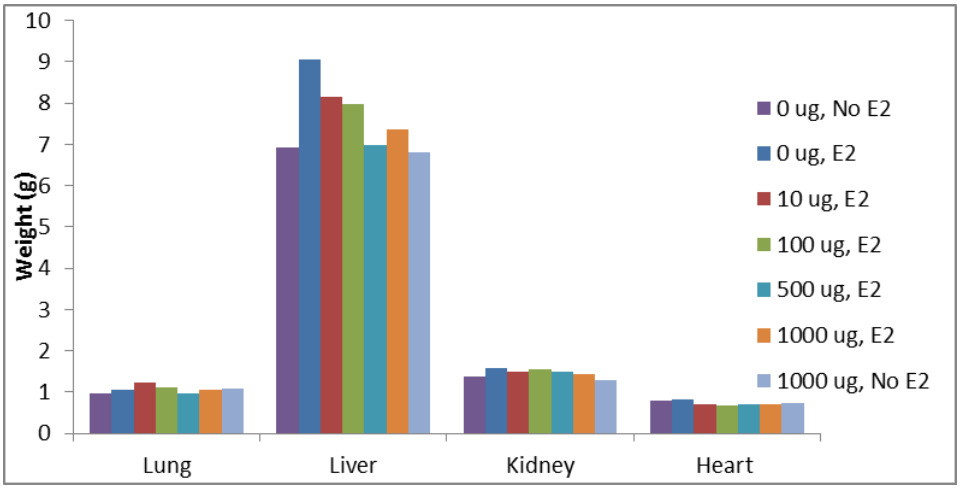


Figure 11. AFPep does not affect organ weights. Organs were harvested at autopsy. There are no statistically significant differences between groups for lung, kidney, or heart. There may be an effect on the liver caused by estrogen (blue bar). There are no differences between AFPep treated groups and groups not treated with AFPep.

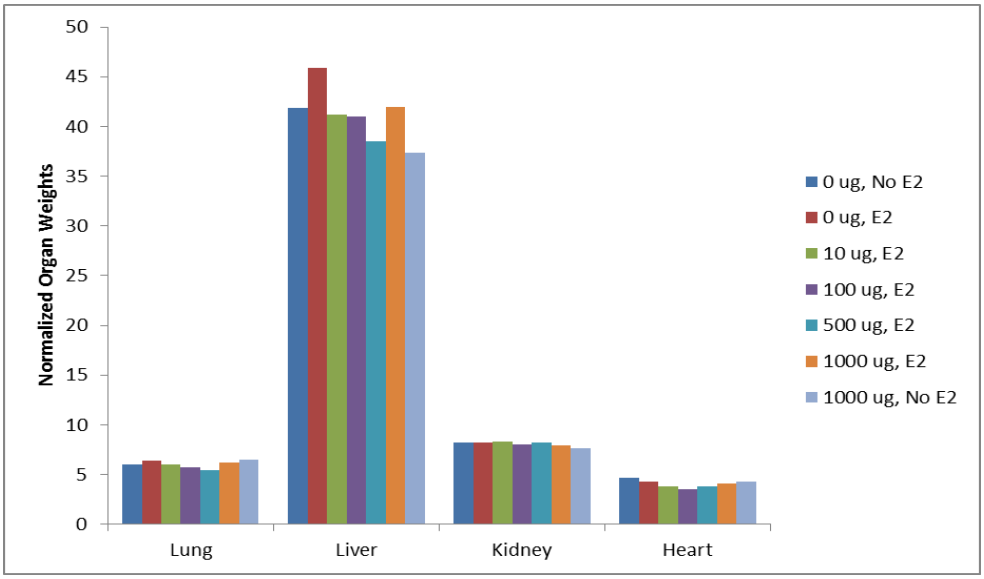


Figure 12. AFPep does not affect organ/body weight ratios. To account for size of the animals, organ weights from Figure 11 were normalized: (organ weight/body weight) x 1000.

Note: We show both organ weights (Figure 11) and normalized organ weights (Figure 12), even though there isn't much difference, because a reader would ask about which ever version were missing if we

Another method to assess potential drug toxicity is to look at histological specimens from various organs, and we used this tool to assess effects of AFPep. Histological analysis can also be used to address a frequently expressed (albeit perplexing) concern from reviewers of our work, namely that AFPep might cause liver cancer. The reasoning for that concern has always been thought to be faulty. Nevertheless, the data from this study should definitively assuage the concern: long-term, high dose (or low dose) treatment of rats with AFPep causes no changes in liver histology. Nothing remotely resembling histologically abnormal tissue can be seen in any of the liver slides prepared from this study. Figure 13 shows slides from liver, heart, and kidney from two animals, one treated with high dose AFPep (Group 7 from Table 1), and one treated only with saline. Neither animal received estrogen. (Not shown are lung or mammary tissue slides, but those will be forthcoming in our next publication). To date, approximately half of the animals described in Table 1 have been subjected to histological analyses, and no evidence can be found of AFPep effects on any tissue.

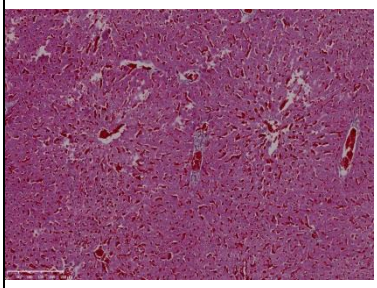
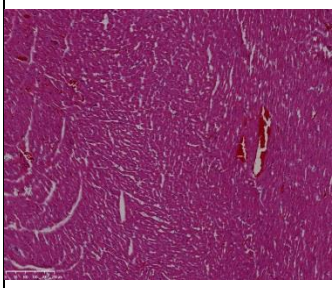
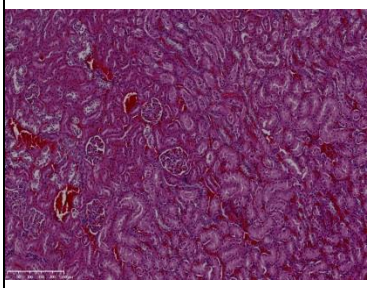
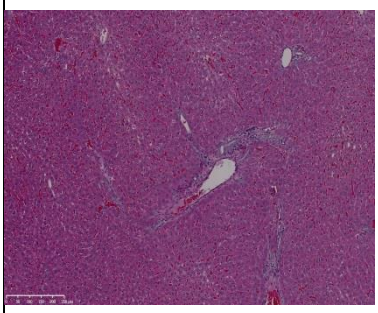
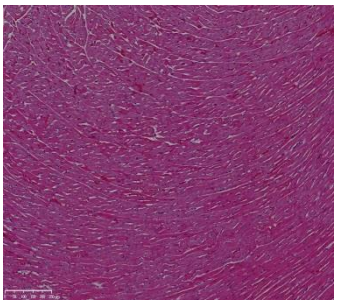
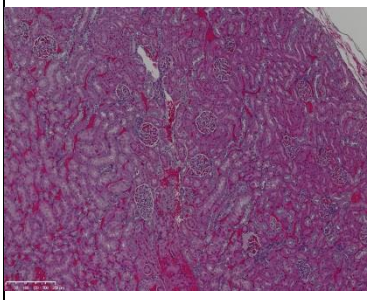
Treatment	Liver	Heart	Kidney
No AFPep			
AFPep			

Figure 13. AFPep has no histologically detectable effect on rat organs. At the time of necropsy, animals from Group 7 High AFPep were compared to those from Group 1 (No AFPep) (groups as defined in Table 1). Organs were harvested and prepared and stained with H&E. One microscopic field from each tissue, and one animal for each group is shown. We have examined hundreds of fields from all tissues from almost half of the animals from each group as of the time of writing of this report. No effect of AFPep is apparent in any tissue.

Daily injections of rats also allowed for continuous monitoring of animal behavior. ACI rats accommodate easily to daily handling; none of the indicators shown in Table 3 suggested any difficulties caused by AFPep.

Table 3: Monitored Parameters in ACI Rats

Posture	Respiratory rate/pattern
Tremors	Grooming
Bizarre behavior	Stool consistency
Rearing	Urination
Alertness	Body tone
Gait	Overall animal reactivity
Piloerection	Body Weights

Comment 3.a Special opportunity to enhance these studies.

In addition to these observations which were a planned activity of Aim 1, there are safety data available from Aim 2 studies which we will mention here. The safety/non-toxicity aspects from Aim 2 studies are from mice, dogs, and monkeys. We have just begun the canine studies; we have extensive studies in mice; the primate studies are completed after substantial accumulation of data. We have never seen signs of toxicity in any of these species.

A unique and time sensitive opportunity arose at our medical center in which 5 monkeys (Macaca mulatta males 11 years in age and 8 kg in weight) became available to us between December 2015 and June 2016 for studies of safety and pharmacokinetics of AFPep. This part of our study was supported entirely by intramural funds ***not DOD-BCRP funds*** *but certainly speaks directly to our hypothesis that AFPep is safe and effective for prevention and treatment of breast cancer. An effective dose of AFPep in mice (4 mg/kg, as determined in studies described below) was converted to an equivalent monkey dose (1 mg/kg) using the conversion chart of Freireich* et al*. and was administered intravenously, subcutaneously or orally to monkeys. There were no changes in any of the clinical, hematological, and serum chemistry parameters listed in Tables 4, 5 and 6 at 4 hours and 24 hours following administration of AFPep. We also obtained pharmacokinetic (PK) data from these primate studies (discussed below).*

***Table 4: Observed Clinical variables in Monkeys* and Dogs**

Heart Rate
Respiration Rate
*O2 Saturation**
*Rectal Temperature**
*Capillary Refill Time**
Mucous Membrane color

*These three parameters were not observed in dogs, since the dogs were not anesthetized. See below for comments regarding canine studies.

***Table 5: CBC Variables in Monkeys* and Dogs**

WBC	*Mean Corpuscular Volume*	*% Neutrophil*
RBC	*Mean Corpuscular Hemoglobin*	*% Lymphocyte*
HGB	*Mean Corpuscular Hemoglobin*	*% Monocyte*
HCT	*Concentration*	*% Eosinophil*
	Autoplatelet	*% Basophil*

***Table 6: Blood Chemistry Variables in Monkeys* and Dogs**

ALP	*BUN*	*Sodium*
ALT	*Creatinine*	*ALB/GLOB Ratio*
AST	*Cholesterol*	*BUN/Creatinine Ratio*
Creatine Kinase	*Glucose*	*Bilirubin-Unconjugated*
GGT	*Calcium*	*Na/K Ratio*
Albumin	*Phosphorus*	*Hemolysis Index*
Total Protein	*TCO2 (Bicarbonate)*	*Lipemia Index*
Globulin	*Chloride*	*Anion Gap*
Total Bilirubin	*Potassium*	*SDMA*
Bilirubin		

With the addition of the studies described above, we can update our Summary of AFPep studies that have shown no toxicity and no side effects. Table 7 is an update from our proposal, with the information arising from this project shown in red font.

Table 7. AFPep treatment studies that showed no toxicity, no side effects.

Species	**Number of AFPep-Exposed Animals**	**Treatment Duration** Days	**Dose, Route** µg/animal/day	**Autopsy Date** Days after Treatment
Mouse	4000	1	1 – 10,000 i.p. or p.o.	1
Mouse	300	30	10 – 100 i.p. or p.o.	1
Rat	1000	23	3 – 300 sc or p.o.	200
Rat	170	200	10 – 1000 sc	1
Dog	7	1	10,000 i.v. s.c. or p.o.	No autopsy
Mouse	5	5	2,000 i.v.	1
Mouse	5	1	10,000 i.v.	5
Primate	11	1	8,000 – 100,000 i.v. s.c. p.o.	No autopsy

Monitoring a wide array of endpoints, no toxicity was seen in the course of a variety of interventions, in four species. This lack of toxicity was not unexpected for four reasons: (1.) AFPep is derived from an indigenous human protein; (2.) AFPep has been designed/parsed/shaped to have only the anti-estrogenic, anti-breast cancer properties of AFP; (3.) The active, anti-breast cancer concentration of AFPep *in vivo* is well below that of the parent protein concentration in the blood of the human fetus; and (4.) The metabolites of AFPep are simple amino acids.

Emphasizing as it does the concept of *efficacy without toxicity*, this project offers substantial encouragement for development of AFPep as a preventive and therapeutic agent. While some believe that all drugs are toxic, we show here that *efficacy without toxicity* is possible and can be achieved in the effort to end breast cancer if we use drugs that are indigenous, natural, and safe (and whose metabolites are equally non-toxic). On the basis of: a.) efficacy in multiple species against xenograft, orthotopic, or spontaneously arising breast cancer tumors (see below), as well as b.) lack of toxicity in multiple species, these studies document clearly that the risks associated with the usual drug development process are minimal for AFPep. The outcomes of FDA-required, GLP-conducted pre-clinical toxicology studies are a foregone conclusion: at the very least, AFPep will have a very wide therapeutic index. Results of Phase I and Ib trials are also likely to be positive: AFPep at therapeutic doses will not be toxic in humans, and will in all probability indicate efficacy (given that AFPep is effective against human breast cancer xenografts).

We conclude from rodent, canine, and primate models that AFPep is efficacious and extraordinarily safe, and should advance to clinical trials for prevention of breast cancer as soon as possible.

Specific Aim 1 Major Tasks 3 and 4 are the subject of Year 2 and 3.

This is the approved Statement of Work for Specific Aim 2:

Specific Aim 2 Demonstrate that AFPep has efficacy against spontaneous, heterogeneous mammary cancer in higher mammals	**Proposed Timeline**	**Progress**	**Comment**
Major Task 1 Establish in normal dogs blood levels of AFPep known to be efficacious against human breast cancer xenografts	Months	Year 2	
Subtask 1 PK of AFPep in mice	1-3	Ongoing	4
Subtask 2 PD – Time to onset and durability of efficacy of AFPep against human breast cancer xenografts	2 - 8	Ongoing	5
Subtask 3 PK of AFPep in dogs	6 - 18	Beginning	6
Milestone(s) Achieved: Half-life, bioavailability and effective blood levels of AFPep in dogs			7
Major Task 2 Demonstrate in dogs with spontaneous mammary cancer that AFPep given systemically induces an anti-proliferative phenotype in the autochthonous cancer prior to its surgical resection			
Subtask 1 In multiple dogs with ER+ mammary cancer, assess biomarker levels in pre- and post-AFPep tumor biopsies	12 - 36	Year 2-3	8
Subtask 2 Present data at national meetings; publish data in peer reviewed journals	24-36		
Milestone(s) Achieved: AFPep proof of efficacy against spontaneous mammary cancer in higher mammals			
Major Task 3 Organize data to revise design of preclinical toxicology and Phase I/Ib clinical trial for future grant proposals			

Comment 4 *Specific Aim 2*, Major Task 1, *Subtask 1* Pharmacokinetics of AFPep in mice

In order to advance a drug to clinical trials, it is essential to assess its pharmacokinetics (PK) and determine a dose and route of administration that yields efficacious blood levels while being well tolerated and convenient for the patient. Our previously reported data in mice and rats indicated that AFPep is effective following oral administration. Obviously, the oral route would be very convenient for patients. Three key questions in this current investigation are:

- What is an efficacious blood level of AFPep?
- Can this blood level be achieved in higher mammals without evidence of toxicity?
- Can this blood level be achieved in higher mammals through the oral route?

Previous studies by our group indicated that between 1 µg and 100 µg was an active dose range following i.p. administration of AFPep in mice. Because the PK of AFPep had never been assessed, we decided to begin our

PK evaluation of AFPep with the higher 100 µg dose, and we evaluated the intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.), and oral (p.o.) routes to determine comparative PK parameters.

Data were fit by non-linear regression (Pharsight Phoenix 64 WinNonLin) to first order pharmacokinetic models representing intravenous bolus dosages or extravascular (1^{st} order absorption and output for intraperitoneal, subcutaneous or oral) dosages. Primary parameters estimated included rate constants for absorption, elimination, and volume of distribution with secondary parameters of maximal concentration, time of maximum concentration, half time and area under the curve.

As shown in Figures 14 and 15 and Table 8, the half-life of AFPep in the circulation of mice is relatively short (6 to 25 minutes, depending on route) but not unusual for peptide-based drugs. The blood level of AFPep is highest after i.v. delivery and lowest following p.o. delivery. The s.c. bioavailability is quite good, being in the 80 % range. Comparison of Area Under the Curve (AUC) for the i.v. and oral routes (see Table 8) indicate that the bioavailability of AFPep by the oral route is rather low, approximately 2%. Nevertheless, this is apparently sufficient for biological activity in mice (as shown later in Figure 18).

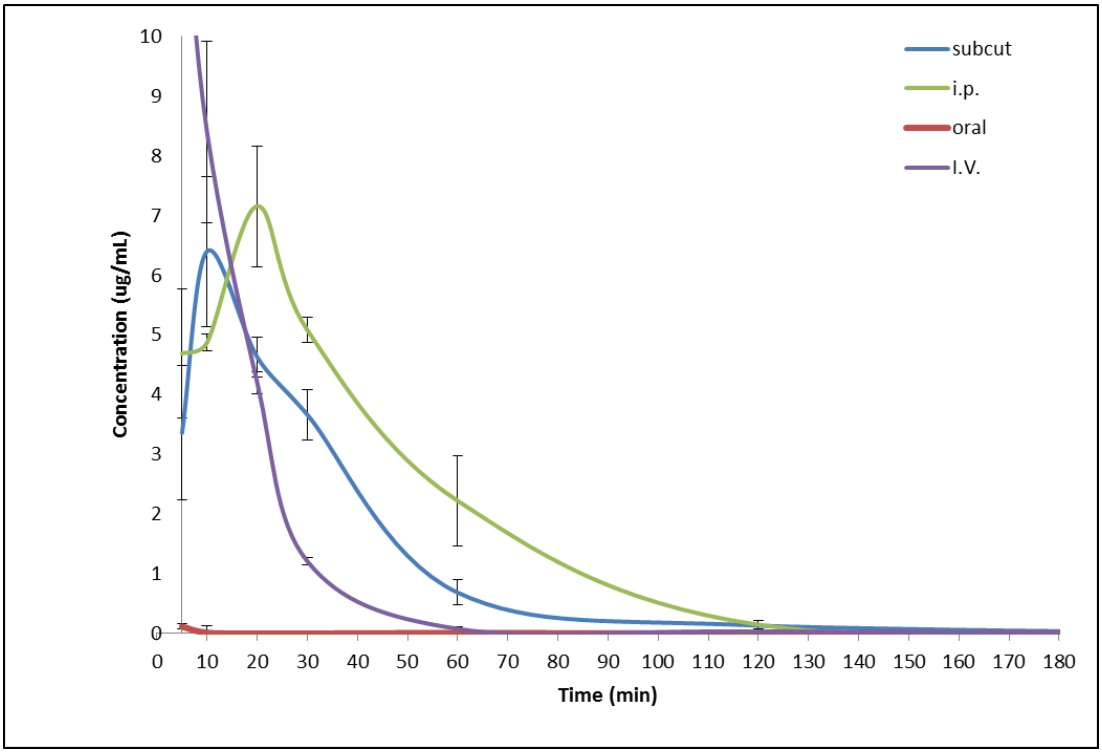


Figure 14. Blood levels and PK of AFPep can be measured in mice. Female Swiss mice (6-9 weeks old, ~ 25 g in weight) were injected with 100 µg of AFPep using the routes indicated. At various time points after AFPep administration, mice were euthanized and exsanguinated. Plasma was obtained and assessed for AFPep levels using LC-MS/MS. The data at each time point represent the mean AFPep plasma level ± the standard error of 3 replicate mice.

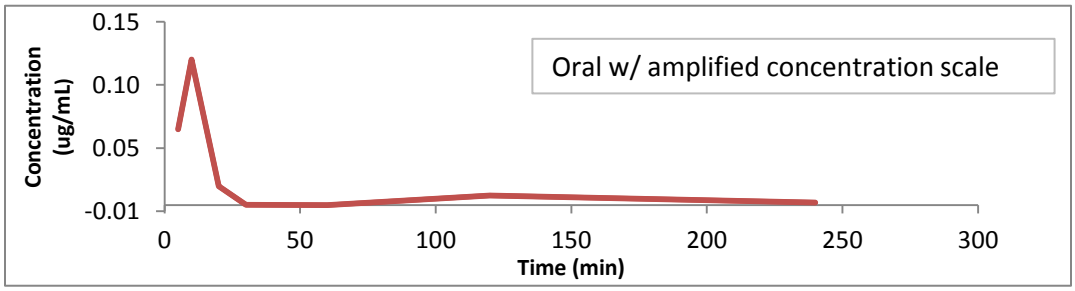


Figure 15. Pharmacokinetics of orally delivered AFPep in mice. The amplified concentration scale facilitates analysis of the oral route of administration. Methodology as in Figure 14.

Table 8: PK parameters of AFPep (4 mg/kg; 100 µg/mouse) given to Mice through various routes				
	I.V.	**I.P.**	**S.C.**	**P.O.**
C_{max} (µg/mL)	12 ± 2.3	7.5± 0.7	6.4± 1.25	0.14 ± 0.1
T_{max} (min)	5 ± 2	15 ± 4	13 ± 3	8.8 ± 5.3
$T_{1/2}$ (min)	11.3 ± 1.5	25 ± 13	11 ± 2	6.3 ± 3.6
V_d (mL)	6 ± 1.7	8.7 ± 2.9	8.0 ± 2.0	N.D.
AUC (min)(µg/mL)	252 ± 28	371 ± 64	207 ± 17	4.8 ± 2
Bioavailability %	---	147	82	1.9

As shown later (in Figure 18), a dose response curve of the anti-estrogenic activity of AFPep in immature mice indicates that the minimum dose of AFPep which achieves maximum effect is 1 µg (0.04 mg/kg). We therefore repeated the PK assessment of AFPep using this 1 µg dose given to immature mice, reasoning that this would provide benchmark blood levels that correlate with efficacy ant that could then be targeted in subsequent studies in higher mammals. Results are shown in Table 9 and Figure 16. The C_{max} was 0.9 µg/mL and the AUC was 50 (min)(µg/ml) following administration of 1 µg AFPep to mice.

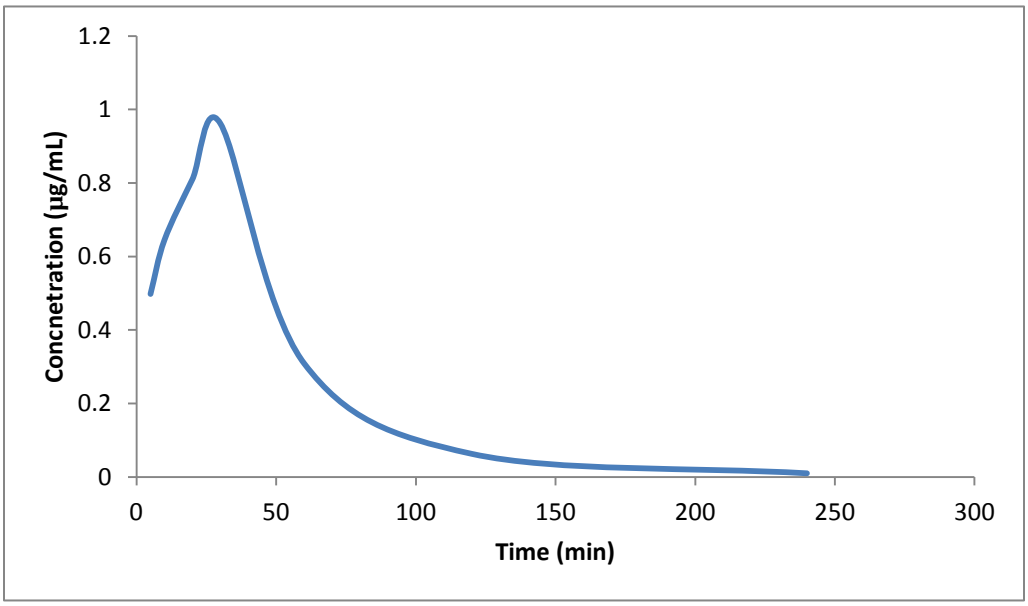


Figure 16. Use of the lowest maximally effective dose of AFPep in mice yields measurable PK parameters. Immature (13-15 day old, ~ 7 g in body weight) Swiss female mice were injected i.p. with 1 µg of AFPep. Blood was pooled from 3 replicate mice at each time point to obtain sufficient plasma volume for assay of AFPep. Data are from one experiment.

Table 9: PK parameters of AFPep (0.04 mg/kg; 1 µg/mouse) given to Mice Intraperitoneally	
	I.P.
C_{max} (µg/mL)	0.962
T_{max} (min)	21
$T_{1/2}$ (min)	15
V_d (mL)	0.4
AUC (min)(µg/mL)	50

In fact, we have already begun the PK studies of AFPep in higher mammals (primates and dogs) and have shown in preliminary work that these benchmark blood levels of AFPep are readily achievable with no evidence of toxicity.

*As mentioned earlier we had a unique time-sensitive opportunity to study AFPep in monkeys at no cost to the DOD-BCRP grant. The monkey work was funded intramurally. We started with the high end of effective mouse doses (4 mg/kg) and converted this to equivalent monkey dose (1 mg/kg). This dose was administered using the intravenous or oral routes. As shown in Table 10 and Figure 17, the i.v. route yielded an average C_{max} of 7 µg/mL and an area under the curve of 254 min*µg/mL. These values are within the efficacious blood level range reported earlier (Fig. 16, Table 9) for immature mice. There was no evidence of toxicity for the primates (as shown in Tables 4-6). The $t_{1/2}$ of AFPep was 68 min which is substantially longer than that found in mice. Very low and inconsistent blood levels of AFPep were found following oral administration of AFPep to monkeys (less than 0.1 ug/mL).*

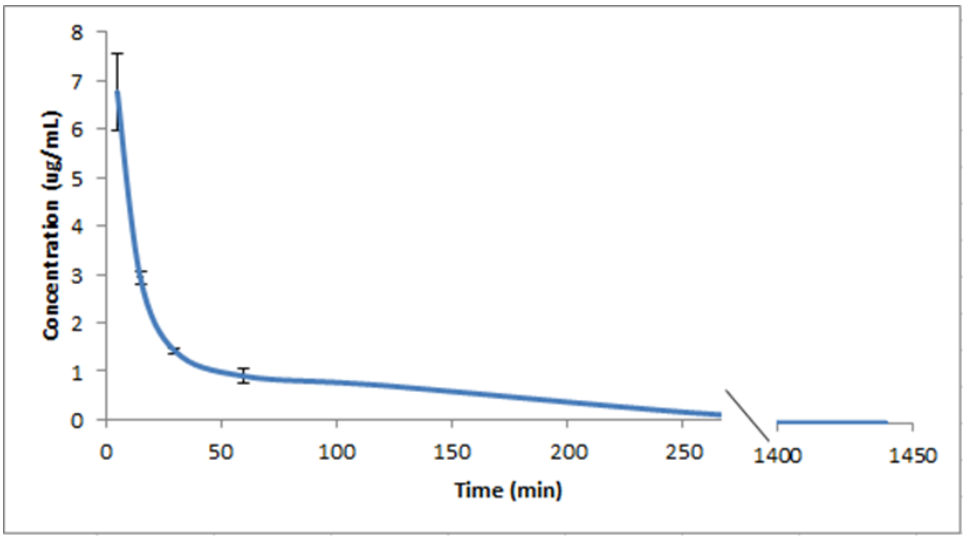


Figure 17. Efficacious blood levels of AFPep can be determined in monkeys following i.v. administration. *Monkeys (Macaca mulatta) were sedated and anesthetized using atropine, ketamine, Midazolam, isoflurane and nitrous oxide. The left cephalic vein was catheterized and used for fluid balance, i.v. administration of AFPep, and for obtainment of blood samples. Oral administration of AFPep was through a gavage tube inserted through the mouth, esophagus, and into the stomach. Blood was obtained at multiple time points after AFPep administration. Plasma was derived and assessed for AFPep levels. Data represent the mean AFPep level ± standard error in three replicate studies.*

Table 10: PK parameters of AFPep (1 mg/kg) given to Monkeys by the intravenous route of administration	
	I.V.
C_{max} *(µg/mL)*	*7.47 ± 2*
T_{max} *(min)*	*5 ± 2*
$T_{1/2}$ *(min)*	*68 ± 8*
V_d *(L)*	*3 ± 1*
AUC (min)(µg/mL)	*254 ± 22*
Bioavailability	*----*

For oral administration, AFPep blood levels were found in only 1 of 3 monkeys and therefore are not reported here.

*The dose of AFPep was escalated to 4 mg/kg in monkeys. The C_{max} from this dose i.v. was 14 µg/ml and the AUC was 1574 min*µg/ml (Table 11). Again, no evidence of toxicity was detected in the monkey after this dose of AFPep. We then tried the s.c. route at 4 mg/kg and found effective blood levels and very good bioavailability (~90 %) (Table 11). When this 4 mg/kg dose was administered orally to monkeys, consistent but low blood levels of AFPep were observed; they were well below the expected efficacious blood level of 0.9 µg/ml (Table 9). However, the persistence of orally administered AFPep in the monkey circulation is really quite impressive and will be reassessed in dogs in Year 2.*

Table 11: PK parameters of AFPep (4 mg/kg) given to Monkeys through various routes of administration			
	i.v.	***s.c.***	***p.o.***
C_{max} *(ug/mL)*	*13.7*	*8.2*	*0.03*
T_{max} *(min)*	*5*	*38*	*214*
$T_{1/2}$ *(min)*	*107*	*27*	*810*
V_d *(L)*	*3.7*	*2.9*	*3.9*
AUC (min)(ug/mL)	*1574*	*1407*	*30.9*
Bioavailability %	*---*	*89*	*1.9*

Although the studies in dogs will take place primarily in Years 2 and 3 of this grant, a preliminary PK study of AFPep in dogs has just been evaluated. Please see Comment 6 on page 24 below.

Conclusions from these PK studies are that:

- **an efficacious blood level of AFPep is approximately 1 µg/mL**
- **this blood level can easily be achieved in higher mammals with no evidence of toxicity.** Primate data shown here, canine data to be discussed below)

This blood level (1 µg/ml) may be achievable in higher mammals by the oral route with further dose escalation, or by use of excipients, enteric capsules, controlled release by hydrogels or other formulation strategies used commonly by pharmaceutical companies. In addition, the subcutaneous route is a viable backup for our canine studies in Year 2 if the oral route continues to yield low blood levels of AFPep.

Comment 5 *Specific Aim 2*, Major Task 1, *Subtask 2* Pharmacodynamics: Efficacy of AFPep against human breast cancer xenografts.

AFPep is designed to be a therapeutic agent as well as a cancer preventive agent. It is important to document the therapeutic efficacy of AFPep, and for this purpose we use a screening assay (because it has a very rapid turn-around time) and a tumor xenograft assay. The screening assay is inhibition of estrogen-stimulated growth of uterus in immature mice; the xenograft model is human MCF-7 cells growing in mammary fat pad of SCID mice. Since the intended route of administration for humans will be oral, we document efficacy in three routes.

Figure 18 shows activity of AFPep using our screening bioassay following three routes of administration. The data demonstrate a broad effective dose range of AFPep, between 1 and 100 µg (0.14 to 14 mg/kg of AFPep in the 7 g immature female mice). No significant loss of biological activity was seen when administering this drug through the oral route, even though the oral route yields substantially lower blood levels compared the i.p. and s.c. routes (as shown in earlier). It is possible that AFPep is active below the predicted blood level of 1 µg/ml. We will investigate further the relationship of pharmacokinetics and pharmacodynamics of orally administered AFPep in Year 2.

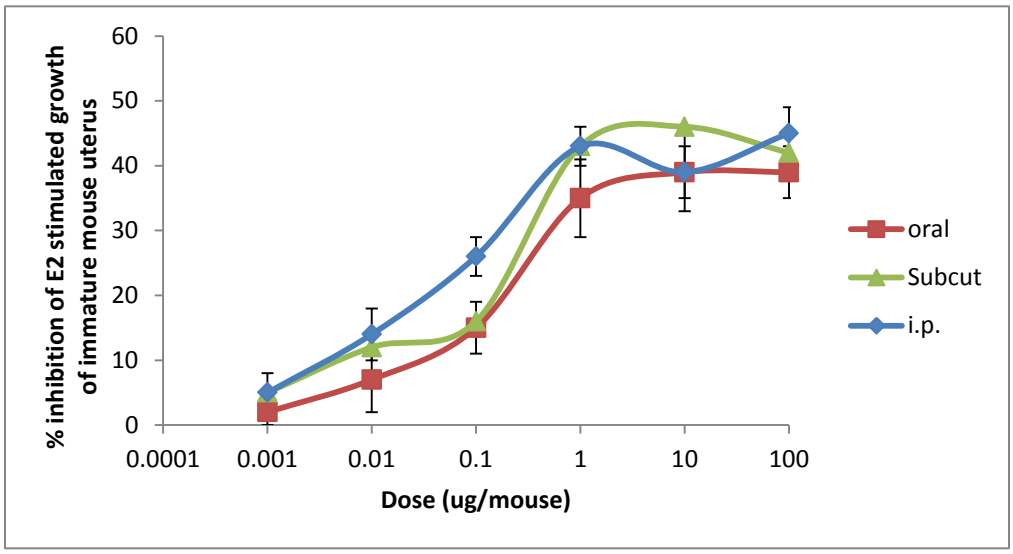


Figure 18. AFPep is fully efficacious after i.p., s.c., or p.o. administration. Various doses of AFPep were injected using the indicated routes into immature (13-15 day old) Swiss female mice. One hour later mice were injected with either saline or 0.5 µg of estradiol. Twenty-two hours later mice were euthanized, their uteri were excised and trimmed free of mesenteries and weighed immediately. The uterine weights were normalized to mouse body weight. There were 5 replicate mice per group. The mean normalized uterine weight ± standard error was calculated. Growth inhibition was calculated using the formula: Growth Inhibition (%) = [(Full estradiol stimulation – estradiol stimulation in test group)/(Full estradiol stimulation – No estradiol stimulation)] x 100 The similarity of the dose-response curves is encouraging and enabling data that bodes well for oral availability in humans.

Figure 19 shows the efficacy of AFPep (100 µg/mouse injected i.p. once daily) against human tumor xenografts. AFPep stops the growth of human MCF-7 breast cancer xenografts growing orthotopically in the mammary fat pad of SCID mice. AFPep is not designed to be a cytotoxic agent that kills cancer and non-cancer cells. Rather, it is intended to be a stasis-inducing agent that keeps cancer in suspended state indefinitely. As can be seen in Figure 19, AFPep is very effective at inducing stasis in human tumors. In earlier studies, we also showed that AFPep is effective against tamoxifen-resistant forms of ER+ MCF-7 breast cancer. Thus, AFPep is very attractive as a therapeutic agent, for the treatment of breast cancer, just as it is as a preventive agent. These data are very encouraging for women who encounter a diagnosis of ER+ breast cancer, in that AFPep could become first-line therapy. AFPep has no known side effects, which is a big improvement over tamoxifen.

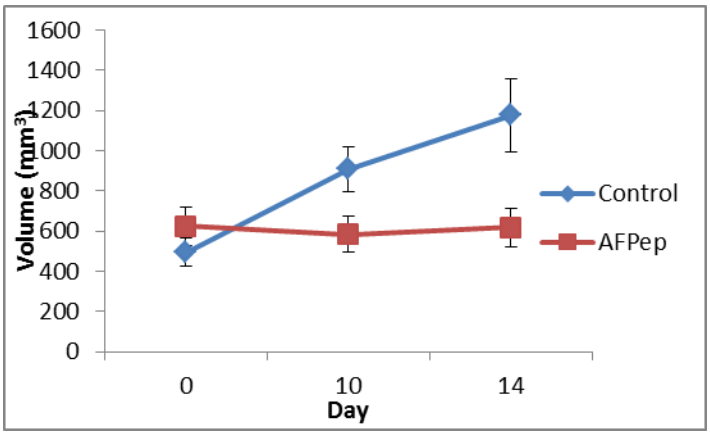


Figure 19: AFPep stops the growth of human tumors growing as xenografts in mice. MCF-7 human breast cancer cells were injected orthotopically into the mammary fat pad of 6 – 8 week old female SCID mice. When tumors reached 5 – 7 mm in average diameter, mice were randomized into the treatment or control group. Tumor was biopsied for biomarker assessment (see Comment 8). Mice were then treated with either saline or 100 µg of AFPep (~ 4 mg/kg) injected i.p. once daily for 14 days. Tumor was measured once daily and was biopsied again immediately after the last treatment on day 14. Points represent average tumor volume ± standard error of 5 replicate mice.

We can conclude that AFPep is effective for the treatment of ER+ breast cancer, and should proceed to clinical trials in humans as soon as possible.

Comment 6 *Specific Aim 2*, Major Task 1, *Subtask 3* PK of AFPep in dogs

We have just begun our pharmacokinetic studies in dogs. The final data will be reported more fully in our Year 2 progress report. However, our preliminary data are shown in Figure 20 and Table 12. AFPep (4 mg/kg) was injected i.v. into female dogs. The C_{max} was 35 µg/ml and the AUC was 900 (min)(µg/ml). The C_{max} and AUC compare very favorably to that found in primates. The $T_{1/2}$ is similar to primates and much longer than that found in mice. In spite of these higher PK values, no evidence of toxicity was seen, as assessed by the parameters of Tables 4 – 6. These data clearly elevate this project to the next level of clinical translation.

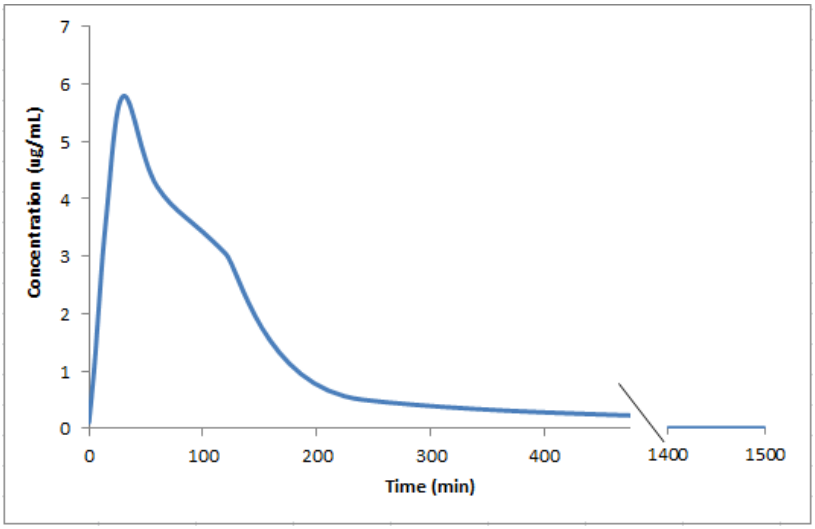


Figure 20. Blood levels of AFPep in canine plasma. AFPep (32 mg) was administered via the s.c. route, and blood samples were taken at various times thereafter. Blood levels substantially higher than the minimally effective dose in rodents are established here, and there were no indications of side effects or toxicity in the dog. This is data from one dog; ongoing studies will document the PK of AFPep in dogs more fully.

Table 12: PK parameters of AFPep (4 mg/kg) given to Dogs by the intravenous route of administration	
	I.V.
C_{max} *(µg/mL)*	*35*
T_{max} *(min)*	*15*
$T_{1/2}$ *(min)*	*18*
V_d *(L)*	*0.9*
AUC (min)(µg/mL)	*900*
Bioavailability	*--*

Prior to issuing a license for an investigational new drug, the FDA requires dose escalation of the drug, either to toxicity levels in higher mammals **or** to a default dose of either 1000 mg/kg *or* 50 times the efficacious blood level. We cannot demonstrate toxicity levels of AFPep (which is a very good thing), so the default doses need to be selected. The 1000 mg/kg dose requirement (~ 10 grams of AFPep in each 10 kg dog) would be very expensive in terms of drug purchase. Fortunately, we have learned through our early canine studies that 50 µg/mL (which is 50 times the efficacious blood level, see discussion in Comments 4 and 5) can be achieved in dogs with no evidence of toxicity. We achieved this blood level in dogs using an i.v. dose of 4 mg/kg/dog. These data will result in significant cost savings as we move into FDA-required preclinical toxicology studies, to be supported by the next grant. These data obviously bode well for the claim that AFPep will be exquisitely well tolerated in humans.

Comment 7 *Specific Aim 2*, Major Task 1 Milestones. Half-life, bioavailability, and effective doses.

On page 18 of this report, we asked three key questions. These studies provide the answers.

Three key questions are:

- What is an efficacious blood level of AFPep? **an efficacious blood level of AFPep is approximately 1 µg/mL**
- Can this blood level be achieved in higher mammals without evidence of toxicity? **This blood level can easily be achieved in mice, rats, dogs, and primates with no evidence of toxicity**
- Can this blood level be achieved through the oral route? **Currently, this blood level can be achieved following oral administration to rodents. In dogs, it may be necessary to modify the formulation of AFPep for oral use.** It is clear that these studies can move forward in dogs using the s.c. route.

Comment 8 relating to *Specific Aim 2*, Major Task 2, *Subtask 1:* Assess biomarker levels.

Previous studies by our group have shown, using Western blots, that significant changes in biomarkers can be detected in AFPep-treated MCF-7 breast cancer xenografts (recapped in Table 13).

Table 13. Effect of AFPep on Biomarkers in Breast Cancer

Biomarker	Change from Control, %
pERα	- 48 ± 5
pERβ	110 ± 15
Rb	38 ± 4
P21	82 ± 9
PCNA	- 53 ± 4

Tumor tissue was harvested for biomarker determination 35 days after tumor transplantation and biomarkers were assayed by Western blot. Change in band density in AFPep-treated mice relative to control group is reported. Mean ± SE from three replicate mice per group. These biomarkers can also be assessed by immunohistochemistry.

A goal of our current study is to assess time-to-biomarker-change and durability of biomarker change induced by AFPep and to develop technology to use immunohistochemistry (IHC) in addition to Western blots. Both time-to-change and durability are important to measure as we move into canine studies and eventually into human studies. It will be important to know how long it is necessary to treat with AFPep before an anti-proliferative biomarker change becomes evident, so that a physician can discern whether AFPep treatment is being effective. It is important to know how long the biomarker change lasts so that biopsies can be obtained with assurance that the drug is still working. That is to say, if the biomarkers fell off to baseline levels within a few minutes of the last AFPep treatment, it would be very limiting in terms of monitoring efficacy. Also, since clinicians usually use immunohistochemistry (IHC) rather than Western blots, it is helpful to ascertain if the biomarkers we have developed using Western blots can also be used in the clinical setting.

Biomarkers were evaluated (from MCF-7 xenografts growing in SCID mice) by immunohistochemistry (IHC) by comparing biopsied tumor samples pre- and post-AFPep. Biopsy samples for these studies were taken from animals used for demonstration of inhibition of xenograft growth shown in Figure 19. Pre- and post-treatment biopsy samples of MCF-7 tumors growing as xenografts in SCID mice were taken from the saline-treated group and the AFPep-treated group, immediately placed in formalin and submitted to our Pathology collaborators for processing and assessment. Slides were read under the direction of Dr. Jeffrey Ross, Chair of our Pathology Department. The following biomarkers were evaluated by IHC: phosphorylated estrogen receptor α (ER α), p21, p53, Ki67, PCNA. These biomarkers were recommended by our pathologist as commonly used in clinical studies to indicate growth inhibition in a tumor. Change was defined as a 50 % difference in distribution and intensity of biomarker-specific stain between treated and control groups in more than three replicate experiments. Of these five biomarkers, only p21 was consistently elevated as a result of AFPep treatment. This indicates that IHC is not as sensitive as Western blotting, which is well known. Additional assessments of these, or other, biomarkers may allow us to detect changes in some of the other biomarkers that Western blotting can identify. However, even if other biomarkers are not detectable by IHC, the existence of this pattern of biomarker change may be unique for AFPep (as compared to other drugs), and may still be a useful diagnostic tool. Alternatively, we can resort to Western blots in order to monitor a larger panel of biomarkers.

Time-to-biomarker-change was assessed at 3, 7, 10, and 14 days into treatment. The p21 elevation was evident after 10 and 14 days into treatment. It is a little surprising that biomarker change was not detectable for more than a week, given the effectiveness of AFPep in our uterine growth inhibition assay which demonstrates a response within 24 hours. This, again, may be a reflection of the low sensitivity of IHC. After 14 days of treatment with AFPep, the durability of biomarker change was assessed: the elevation of p21 remained at 4 hours, 24 hours, and 48 hours after cessation of AFPep treatment. We have not yet assessed longer durations. Thus, durability of biomarker change is quite long, an asset of AFPep and/or IHC that will allow flexibility in measurements of tumor responsiveness.

It is well known that IHC is not as sensitive as, yet more clinically translatable than, Western blot analysis of biomarker change. It is not surprising that more AFPep-induced biomarker changes were noted with our earlier Western blot analyses. In Year 2 we will assess additional biomarkers using Western blot in control and AFPep-treated tumor tissue in order to add to the assessment value of the p21 biomarker response. The most sensitive changes detected by Western blot will then be assessed by IHC. Thus, a larger menu of biomarkers may be available for our canine studies in which an anti-proliferative effect of AFPep will be assessed in domestic dogs presenting with spontaneous mammary cancer. We are also fully cognizant of the possibility that the biomarker profile in spontaneous canine mammary cancer may be different from that of MCF-7 human breast cancer, so adaptability to a broader menu of biomarkers seems to be a prudent position.

Specific Aim 2 Major Tasks 2 and 3 are the subject of Year 2 and 3.

- **What opportunities for training and professional development has the project provided?**

Although it was not the intention of this award to promote training, we have, in fact trained several individuals and enhanced their professional skills and development.

- Ms. Wasila Mansouri, a biomedical engineering alumna of Rensselaer Polytechnic Institute, has been trained in the scientific method of modern biological sciences and has enhanced her professional skills substantially. Her career path will lead her to medical school and to a career as a physician scientist, and she will be substantially more competitive than before she worked on this project.
- Three medical students have been trained in the scientific method, and will continue their training to the point that they will earn their M.D. with Distinction in Research.
    - Mr. Tanuj Sharma has been using the safety studies to learn the scientific method, and will present posters and write a thesis on this work.
    - Ms. Amber Quave has been using the pharmacokinetic studies to learn the scientific method, and will present posters and write a thesis on this work.
    - Mr. Casey Hladik has been using AFPep on some pioneering work (funded by our Department of Surgery) related to glioblastomas, and will write a thesis on his work.
    -
- Two medical residents have been working with us.
    - Dr. Cassandra Denefrio has been working on a project related to the use of AFPep in treatment of uterine fibroids (funded by our Department of Obstetrics and Gynecology), and she has substantially enhanced her professional skills.
    - Dr. Alexander Riccio has been working on a project related to the use of AFPep in treatment of glioblastoma, and directing a student (Mr. Hladik) in this effort.
- One undergraduate student, Mr. Christopher Sullivan (a sophomore at University of Buffalo, NY) has been working with us and intends to enhance his competitiveness for post-graduate training.

- **How were the results disseminated to communities of interest?**

One publication appeared in a book on AFP:

Bennett, JA, Jacobson, HI, and Andersen, TT. Anti-Breast Cancer Drug Derived from Alpha-Fetoprotein. In Alpha-Fetoprotein Functions and Clinical Applications, Lakhi, N. and Moretti, M. eds, Nova Science Publishers, Inc. New York, 2016.

Discussions with Breast Cancer Consumer Advocates led to the invitation to three advocates to join our team of investigators. Ms. Jocelyn Banks (from the "Mommy Has Breast Cancer" foundation), Ms. Monica Vakiner (representing the Cancer Resource Center of the Finger Lakes), and Ms. Joan Isman (now living in Maryland) have joined our team. They share our recent outcomes with their local breast cancer communities, and have agreed to work with us in the future.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the next year, we plan to accomplish **Aim 1 Major Task 4, Subtasks 1 through 4** which is to determine the minimal duration of treatment of ACI rats with AFPep and still yield life-long protection. This is a change of the sequence of Major Tasks, and will require written request of the CDMRP. The need for this change was made paramount by the very hopeful and surprising studies outlined earlier in this proposal, in which it appears

that very brief treatment, with very low doses of AFPep, are optimal for the prevention of breast cancer. This exciting observation must be confirmed as soon as possible.

Aim 1 Major Task 3, **Subtasks 1 through 4**, namely to demonstrate interdiction of breast cancer at various stages of disease progression, will be accomplished in Year 3. We will use the ACI rat model for this objective.

In the next year, we plan to complete Aim 2 Major Task 1 Subtask 3 which is the assessment of PK in dogs, and to begin **Aim 2 Major Task 2**, **Subtask 1**, namely to demonstrate in dogs with spontaneous mammary cancer that AFPep given systemically will induce an anti-proliferative phenotype.

In the next year, we plan to accomplish **Aim 2, Major Task 2, Subtask 2**, namely we will present our results at national cancer meetings so as to enhance awareness of our results and elicit others to work in the area of breast cancer therapeutics related to AFPep. We intend to present our work at the AACR meeting, and encourage our medical student workers to present their posters at regional and national professional meetings.

In the next year, we intend to accomplish **Aim 2, Major Task 3**, namely to organize our data so as to be able to apply for a grant from the DoD CDMRP Breakthrough Level 3 funding to complete FDA-required pre-clinical toxicology of AFPep prior to entry into clinical trials.

Within the next year, we plan to submit 2 or 3 manuscripts for publication.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - While many people believe that all drugs have some toxicity, this project is demonstrating clearly that *efficacy without toxicity* is possible, and can be achieved for the prevention and treatment of breast cancer. We are demonstrating this by using molecules that are indigenous, natural, safe, and effective, and whose metabolites are also non-toxic. Peptides fit that description; AFPep demonstrates those ideals.
- **What was the impact on other disciplines?**
  - Nothing to report.
  -
- **What was the impact on technology transfer?**
  - Nothing to report.
  -
- **What was the impact on society beyond science and technology?**
  - Nothing to report.
  -

5. CHANGES/PROBLEMS: Changes in approach and reasons for change

-
- We intend to do Prevention Trial C before Trial B. This is a change in the sequence of tasks outlined in our approved SOW, and will require prior approval from the Grant Officer (not yet requested). This is especially important in view of the results from the first year of the project. No experiments will be added, none will be deleted; only the sequence of work will be altered.
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- We intend to assess multiple approaches to oral bioavailability in dogs. This is a part of our existing (approved) project. However, canine clinical studies can begin now, using the s.c. route of administration, so those studies will not be deterred.
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- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Nothing to report.
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- **Changes that had a significant impact on expenditures**
  - Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
- **Significant changes in use or care of human subjects** Not applicable
- **Significant changes in use or care of vertebrate animals.**
  - Nothing to report.
- **Significant changes in use of biohazards and/or select agents**
- Not applicable

6. PRODUCTS:

Publications, conference papers, and presentations

One publication appeared in a book on Alpha-Fetoprotein:

Bennett, JA, Jacobson, HI, and Andersen, TT. (2016) Anti-Breast Cancer Drug Derived from Alpha-Fetoprotein. *In* Alpha-Fetoprotein Functions and Clinical Applications, Lakhi, N. and Moretti, M. *eds*, Nova Science Publishers, Inc. New York, 301-318.

The following patents protect the intellectual property associated with AFPep.

1	9,249,189	T	Alpha-fetoprotein "ring and tail" peptides
2	7,964,701	T	Alpha-fetoprotein peptides
3	7,943,577	T	Alpha-fetoprotein peptides and uses thereof
4	7,598,342	T	Alpha-fetoprotein peptides and uses thereof
5	7,220,402	T	Alpha-fetoprotein peptides and uses for imaging
6	7,132,400	T	Alpha-fetoprotein peptides and uses thereof
7	7,122,522	T	Alpha-fetoprotein peptides and uses thereof
8	6,818,741	T	Alpha-fetoprotein peptides and uses thereof

The first listed patent (number 9,249,189) is the most recently issued, and is being prosecuted now as a divisional patent to broaden its coverage. All of these patents are based on scientific accomplishments with CDMRP support, all were prosecuted by the Albany Medical College, and the total cost of filing and maintaining these patents exceeds $400,000 to date.

- **Other Products**

There is nothing to report yet. However, it occurs to us that AFPep could move into veterinary clinical utility, in that dogs are not treated with tamoxifen (because tamoxifen is toxic to dogs) but could be treated with AFPep (because we have shown that it is not toxic in dogs).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

A

Name:	*Thomas Andersen, Ph.D.*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	*Dr. Andersen takes the lead on prevention studies and administers all aspects of the award.*
Funding Support:	*Funding from this award, and from the Albany Medical College*

-

- B

Name:	*James A. Bennett, Ph.D.*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	*Dr. Bennett takes the lead on PK studies*
Funding Support:	*Funding from this award, and from the Albany Medical College*

-

C

Name:	*Herbert I. Jacobson, Ph.D.*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	*Dr. Jacobson advises on endocrinology-related aspects of the project.*
Funding Support:	*Funding from the Albany Medical College*

D

Name:	*Paul Feustel, Ph.D.*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	*Dr. Feustel is the statistician on this project.*
Funding Support:	*Funding from this award, and from the Albany Medical College*

E

Name:	*Wasila Mansouri*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	*Ms. Mansouri is involved in all aspects of the project.*
Funding Support:	*Funding from this award.*

- F

Name:	*Qishan Lin, Ph.D.*
Project Role:	*Consultant (not paid)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.3
Contribution to Project:	*Dr. Lin manages a core facility for mass spectroscopy analysis of PK samples*
Funding Support:	*Employed at University at Albany*

- G

Name:	*Douglas Cohn, DVM*
Project Role:	*Veterinarian*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.1
Contribution to Project:	*Dr. Cohn participates in the non-human primate studies*
Funding Support:	*Funding from the Albany Medical College*

-

- H

Name:	*Jeffrey Ross, M.D.*
Project Role:	*Pathologist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.1
Contribution to Project:	*Dr. Ross supports the biomarker analyses*
Funding Support:	*Funding from the Albany Medical College*

-

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

The Albany Medical College invested $25,000 in the AFPep project so that we could obtain PK data in non-human primates. This investment did not slow or alter our progress toward completion of the project goals, but it did enhance markedly our PK studies and ability to develop AFPep toward clinical utility.

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- **What other organizations were involved as partners?**

No other organizations were involved as partners. We used a contract research organization (Proteomics Core at University at Albany) for mass spec analyses, and another CRO (AmbioPharm, Inc.) for synthesis of AFPep, but these were fee-for-service arrangements, not partnerships.

8. SPECIAL REPORTING REQUIREMENTS

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- Noting to report.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

We have appended the manuscript of our recently published book chapter.

Anti-Breast Cancer Drug Derived from AFP

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[2]Department of Obstetrics & Gynecology

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Abstract

We will review the clinical and laboratory investigations that suggested the anti-estrogenicity of AFP, as well as the epidemiological studies that linked AFP to reduced breast cancer incidence, and we will articulate the concepts associated with using truncated segments of AFP for the control of breast cancer. In terms of drug development, we will review the generalizable means by which the anti-breast cancer activity of AFP was identified, isolated in the third domain of the molecule, and developed into an orally active cyclic nine amino acid peptide (AFPep). We illustrate the properties of AFPep, emphasizing its efficacy and lack of toxicity. Looking forward, we articulate the potential of AFPep for the treatment and prevention of breast cancer, as well as speculate about its utility for other clinical challenges. We conclude that the development of the homobiotic peptide AFPep may serve as a prototype for the development of other pharmaceutical molecules derived from molecules of human physiology.

Introduction

Many putative functions have been ascribed to α-fetoprotein (AFP) (1;2), including fatty acid transport (3), immune regulation (4), and anti-estrogenicity. (2) The teleological concept of AFP possessing an anti-estrogenic effect to protect a fetus from maternal estrogen (5) rests on the recognition that the fetus is exposed to high levels of estrogen (6;7) and has estrogen receptors (8) but does not respond to estrogen in the expected manner (9). Few other components of the antenatal milieu could offer such protective activity. Further, the AFP gene is substantially down-regulated shortly after birth (2), coincident with the drop in estrogen levels in neonate tissues. (7) We have long been interested in the anti-estrogenic effects of AFP (10), with a particular goal of developing molecules that might be useful in clinical situations that call for anti-estrogens, notably including treatment of breast cancer. (11) The purpose of this contribution is to describe the rationale and strategies by which we developed an anti-breast cancer drug based on the structure of AFP and suggest this approach may be generalizable for some of the other putative functions of AFP (or other natural molecules). The importance of developing pharmaceutical agents that are homobiotic can be summarized in the phrase "efficacy without toxicity" (12). It may be that overuse of the word "natural" in both the scientific and lay literature has led to loss of meaning or even specificity. Taxols, for example, are natural products but their role in human physiology is certainly xenobiotic and might be expected to be accompanied by toxicity. On the other hand, homobiotic replacement agents such as hormones and hematopoeitic factors are generally effective and reasonably well tolerated. It makes sense that a molecule (or an analog of a molecule) that is indigenous to human physiology might be expected to be well-tolerated (*i.e.*, exhibit no toxicity) and effective since it would

utilize the normal signaling pathways. Furthermore, isolating from a natural molecule the structural component that possesses only one desired function of that molecule should improve selectivity and tolerability. We surmised that homobiotic analogs of AFPep might be able to capture specific functions of AFP and generate the single desired response without the complications that would be associated with introduction of the intact AFP molecule. We describe here the implementation of that strategy to AFP and the subsequent development and characterization of a small homobiotic peptide which we call AFPep, and we illustrate why it is safe and effective for the treatment and prevention of breast cancer.

AFP is anti-estrogenic

That AFP was involved in estrogen-modulated processes was suggested by clinical observations many years ago. AFP was found to be produced by the liver. (13) Amenorrhea was identified as an early symptom of hepatoma (over-production of AFP) (14), and partial hepatectomy of the tumor-involved liver lobe brought about reversal of both amenorrhea and galactorrhea (15). Nerad and Skaunic reported a patient who became pregnant after liver lobectomy to remove hepatoma (16). AFP produced by hepatoma or gastric cancer serves as a biomarker for those diseases (13;17;18), though, of course, there are no reports to suggest that AFP causes those diseases. Soto and Sonnenschein (19) reported on the role of AFP in controlling growth of estrogen-sensitive cells. Despite the presence of estrogen receptors in fetal, infant, and child tissues (8), fetal tissue does not respond to estrogen (7-9). Serum AFP levels are high (mg/mL) during fetal life and decline rapidly after birth to ng/mL. (20;21) These reports from the clinic suggest that AFP interferes with estrogen-induced responses, and this became important in the breast cancer arena because of the numerous reports that suggest overexposure to estrogen promotes development of breast cancer (22).

In 1979, laboratory studies began to report on the probable role of AFP in growth inhibition of estrogen-sensitive tumors in newborn rats. (23) Soto and Sonnenschein (19;24;25) showed that estrogen-independent tumors implanted into newborn or adult rats grew without delay and without respect to added estrogen. However, estrogen-dependent tumors grew promptly in adult animals but experienced a delay of several days when inoculated into newborn rats. Their interpretation was that AFP present only in newborn animals was responsible for the inhibition of tumor growth (26). These authors also showed that purified AFP had growth-inhibiting effects against estrogen-sensitive cell lines in culture (19). Other endpoints reflected similar suggestions for the anti-estrogenic activity of AFP, one example being that uterine wet weight and stimulation of uterine epithelial mucosa were significantly decreased in rats bearing an AFP-secreting hepatoma (27).

An elegant series of studies highlighted the role of AFP in breast cancer (19;26). Rats already bearing an estrogen receptor-positive breast cancer were implanted with a hepatoma cell line that secreted large quantities of AFP, or with a hepatoma cell line that did not secrete AFP. Mammary tumors actually decreased

in size in animals receiving the AFP-secreting hepatoma cell line, but not in animals that received the control hepatoma cell line. Similar results were obtained by injecting partially purified preparations of AFP. AFP purified from cultured human hepatoma cells inhibited the growth of human breast cancer xenografts (including MCF-7 and T47D lines) growing in SCID mice (28), emphasizing the importance of continuing to develop AFP as a pharmaceutical entity to combat breast cancer. Historically, that development process included a progression in the purity of AFP, as technology allowed. Originally, studies used animals bearing AFP-secreting hepatomas (26;27), then AFP preparations purified from rat fetuses (19) or from human umbilical cord blood (29), from rat amniotic fluid (10), or from culture media of human hepatoma cells (28), and eventually from a bacterial expression system (30). As described below, further development included chemical synthesis of analogs of AFP, assuring that the anti-cancer activity is associated with AFP, not with any putative contaminant molecule.

A recurring observation in laboratory studies was that AFP could undergo a change in conformation in the presence of hydrophobic ligands such as estrogen (31) and fatty acids (3), and that this conformational change would increase its anti-estrogenic activity. (31) For example, difference spectroscopy studies indicated that AFP undergoes a conformation change in a molar excess of estrogen.(31) Coincidentally, AP's antiuterotrophic activity is increased when it is in the presence of a molar excess of estrogen.(10) The suggestion was made (3;31) that there was an active anti-estrogenic site in AFP, an 'epitope' which could become more available to growth regulating receptors depending on the molecular environment in which AFP found itself. This observation, too, encouraged further development of that 'epitope,' but it also led to the conclusion that the obvious mechanism of simple binding and sequestration of estrogen by AFP is insufficient to explain the anti-estrogenic activity of the protein, especially considering that adding a molar excess of estrogen could not overcome the growth inhibitory effect of AFP. (10)

Epidemiology studies suggest AFP modulates breast cancer

Epidemiological data suggest that AFP is one of the factors in pregnancy that confer on parous women their significant reduction in risk of breast cancer. As shown in Table I, AFP is elevated in maternal serum during pregnancy. (28) Furthermore, there are various conditions associated with pregnancy (such as maternal race, weight, consumption of alcohol, hypertension, number of fetuses in utero, or neural tube defect in the fetus) in which the maternal serum AFP concentration [MSAFP] is substantially altered from normal pregnancy levels. In studying the literature, Jacobson noted (32) the consistent and striking correlation that in those pregnancy conditions associated with an elevated level of MSAFP (e.g., race and weight), there was a significant reduction in the lifetime risk to the mother of acquiring breast cancer. Conversely, in pregnancy conditions characterized by low MSAFP (e.g., alcohol use), subsequent breast cancer risk was elevated.(28) This led Jacobson and Janerich (33-35) to undertake epidemiological studies of three other pregnancy

conditions (hypertension, twinning, and neural tube defects) that are associated with elevated MSAFP. For each of these conditions, a correlation between high maternal serum AFP and reduced breast cancer risk was observed.(28) Then, too, Ekbom *et al.* (36) published an epidemiological study which suggests that, at least in the case of hypertension during pregnancy, the reduction in breast cancer risk is also passed on to the fetus. All known reports that have failed to confirm this observation have employed small populations and lacked sufficient power to confirm or negate the observation (37-39), or included older populations of women in whom malignant transformations are likely to have occurred after their final pregnancy (38;39). On the other hand, a report by Albrektsen *et al*. (40) using a large population of Norwegian women stated explicitly that the "reduced risk of breast cancer observed among women with multiple births, compared to women with singletons only, is consistent with results reported by Jacobson *et al*. (33). Our results supported this observation and added that the protective effect of twin pregnancy was even more pronounced when it was the last birth (33); (37))."

Table I. Association of High Maternal Serum [AFP] with Decreased Breast Cancer Risk

Maternal Condition 1	Maternal Condition 2	[MSAFP]	Maternal Breast Cancer Risk
Pregnant	Non-pregnant	1>2 (41)	1<2 (42)
Pregnant, Black	Pregnant, White	1>2 (20)	1<2 (43)
Pregnant, lean	Pregnant, obese	1>2 (20)	1<2 (22)
Pregnant, consuming no alcohol	Pregnant, consuming alcohol	1>2 (44)	1<2 (45)
Pregnant, hypertensive	Pregnant, normotensive	1>2 (46)	1<2 (34)
Pregnant with multiple fetuses	Pregnant with a single fetus	1>2 (47)	1<2 (33)
Pregnant, fetus with NTD	Pregnant, fetus without NTD	1>2 (41)	1<2 (35)

NTD is Neural Tube Defect. Numbers in parentheses are references for the data. Redone and corrected from (28).

More recently, Richardson *et al*. (48) employed data from the University of California at Berkeley Child Health and Development Study to measure directly (*i.e.*, in a single cohort) the association between maternal serum AFP concentrations and subsequent breast cancer incidence. They found that the concentration of AFP in maternal sera that had been cryogenically preserved was inversely correlated to the risk of breast cancer in these same mothers 20 to 30 years after their pregnancies. Richardson (48) concluded, "the results of this study agree with the protective effect reported by Jacobson *et al*. (33) and Thompson *et al*. (34) using surrogate indicators (multiple births and hypertension) for a high level of AFP during the index pregnancy." Melbye *et al*. (49) concluded "a high level of AFP in maternal serum during any pregnancy is associated with a low overall incidence of breast cancer, and, in particular, with a low incidence of advanced breast cancer. This association appears particularly strong for a pregnancy occurring at a young age." These studies suggested to us that AFP should be pursued as a modulator of breast cancer (32).

Other workers had proposed various hormones of pregnancy as the agent(s) responsible for the pregnancy-associated reduction in risk of breast cancer. Estriol (50-52), progesterone together with estrogen (53), or human chorionic gonadotropin (54) injected serially into carcinogen-exposed rats decreased the

incidence of mammary cancer compared to carcinogen-exposed animals not treated with those hormones, but there was no understanding of why those various treatments would elicit a similar response. Jacobson *et al*. (55) proposed that these hormones were not the proximal modulators of cancer in the mammary tissue, but rather that they acted on the liver to stimulate production of AFP, and that AFP modulated breast cancer. These workers repeated the experiments involving treatment of carcinogen-exposed rats with progesterone plus estrogen, progesterone plus estriol, estriol alone, or chorionic gonadotropin and observed the previously identified reduction in mammary cancer incidence. However, they also measured serum AFP levels in the animals and showed that in every case, AFP levels were increased by the hormone treatment. An *in vitro* system using human HepG2 cells treated with the hormones of pregnancy elicited AFP, which when applied to MCF-7 cells inhibited their proliferation. Antibody directed against human AFP obviated that inhibition of proliferation, suggesting rather convincingly that, in either animal studies or in women who experience pregnancy, AFP is the proximal agent that inhibits breast cancer (55).

Summarizing, the observations are clear that breast cancer is driven by estrogen, that AFP is an anti-estrogen and is an anti-breast cancer agent. Therefore, it seemed logical to develop AFP as a pharmaceutical agent for the treatment of breast cancer. Of course, an intact protein with as many activities as has been reported for AFP would be undesirable as a pharmaceutical agent. A better approach would be to identify the anti-cancer active site of AFP and develop it into a small molecule, a process which is described below.

Development of AFPep. In overview, an extensive series of parsing studies summarized in (12), employing an anti-estrogenic activity assay (10) (i.e., inhibition of estrogen-stimulated growth of immature mouse uterus) for assessment of intended effect, was used to identify the anti-estrogenic active site of AFP (10;12) which was then developed into a small, readily synthesized, stable, orally active cyclic 9-amino acid peptide referred to as AFPep.

Festin *et al*. (31) used a baculovirus system to express domains and subdomains of AFP and showed that the anti-estrogenic activity was contained primarily in the C-terminal third of the protein. Subsequent efforts to further define the active site employed peptide synthesis of large fragments of this domain (56-58), followed by synthesis of smaller and smaller peptides (59), each time guided by the anti-estrogenic uterine growth inhibition assay. An 8-amino acid peptide that retained essentially full anti-estrogenic activity of the intact AFP molecule was identified (59), whereas smaller fragments had diminished activity (60). This 8-mer peptide had none of the other activities associated with full-length AFP. It was developed further through modeling and rational design approaches (59;61-63), evolving eventually (12) into a small, readily synthesized, stable, orally active cyclic 9-amino acid peptide which we call AFPep (**Figure 1**). (31;59;61-66) AFPep is the anti-estrogenic/anti-breast cancer site in the AFP molecule (57;59;61;65-67); it inhibits estrogen-induced development and growth

of experimental breast cancers (59;61;62;64-66;68). AFPep has none of the other active sites and none of the side effects of AFP such as immune suppression or hepatic growth (4;69;70). AFPep has been found to be active after oral administration in three different assays in two different species in both immature and adult animals for the prevention and therapy of breast cancer (66) (see below).

Using a human breast cancer xenograft assay in mice (65), we found that either AFPep or its parent protein, AFP, given once daily, significantly inhibited the growth of human MCF-7 breast cancer xenografts (28;65). Dose response curves are shown for both entities in **Figure 2**. AFPep (m.w. 970) and AFP (m.w. 69,000) are similar in their potencies in that significant activity is apparent above a dose of 0.0001 μmoles of each agent. AFPep was evaluated against additional ER+ human breast cancer cell lines (T47D and ZR75-1 grown as xenografts) and was found to inhibit their growth (65;71). These important observations (together with those related to efficacy against tamoxifen-resistant cancers, see below) provided impetus for development of AFPep as a therapeutic agent.

As discussed in (12), several important aspects of peptide drug development were incorporated in the process of developing AFPep, but here we wish to emphasize that it is entirely likely that other activities associated with intact AFP could be developed into small molecules using similar approaches. Advantages of peptide analogs of AFP, be they cyclic or linear molecules, include generation of a single activity, utilization of intrinsic pathways, and low toxicity. Specificity of drugs may be more readily achieved by using peptides (as compared to non-peptidic organic molecules) because of the multitude of isosteres available to peptide chemistry; peptides can be exquisitely designed to interact with a specific receptor (72). Moreover, peptides are widely accepted now as a good investment for development by the pharmaceutical industry, and there are more than 60 peptide drugs in current clinical use, capturing a significant market share of the pharmaceutical industry.(73) In the case of AFP, it should be possible, using the parsing techniques discussed herein, to identify individual 'epitopes' corresponding to the desired activity, and so remove all other active sites. As always, a sensitive assay is required to monitor the parsing process. In this instance, an anti-estrogenic assay was utilized but other assays would identify other activities. Once developed, an analog of AFP would be expected to work through the intrinsic pathway used by the parent AFP, and this is likely to minimize off-target effects and perhaps to prolong utility of the drug. Since such a molecule would be using native signaling pathways, it may be less likely to engender chemotherapeutic failure through development of drug resistance. While this concept has not yet been definitively demonstrated, it may be that AFPep will retain efficacy longer than tamoxifen or other agents (see below). Low toxicity is inherently associated with peptide drugs, due to target specificity as well as to metabolite tolerability. The metabolites of AFPep are simple amino acids, and are not toxic. Many non-peptidic drugs get oxidized and become more toxic than the original molecule, a

process that is easy to avoid with peptides derived from parent proteins such as AFP. Thus, there are multiple advantages to this approach in drug development.

AFPep is Useful for Treatment and Prevention of Breast Cancer

AFPep Can be Added to, or Used in Place of, Tamoxifen. Tamoxifen has been the most widely used agent for the treatment of estrogen receptor-positive breast cancers and has provided significant benefits to women (74). However, a vexatious problem encountered in its clinical use is that not all ER+ breast cancers are sensitive to tamoxifen. Moreover, it is not uncommon that women whose disease is being managed successfully by tamoxifen therapy will experience recurrence of cancer apparently because their tumor has acquired resistance to tamoxifen. We considered it important to determine whether AFPep was active against ER+ breast cancer that had become resistant to tamoxifen, so we developed an MCF-7 human breast cancer sub-line that was resistant to tamoxifen, grew it as xenografts, and showed that AFPep was equally effective against wild type and tamoxifen-resistant MCF-7 breast cancer (65). In other studies, AFPep was found to be additive with tamoxifen in its inhibition of wild-type MCF-7 xenograft growth, and in its prevention of MNU-induced mammary cancer (64). Thus, AFPep may be used as a replacement for tamoxifen, or at the very least, be used after tamoxifen has failed. But AFPep may be even better than that. First, an important observation is that AFPep actually inhibited the uterine hyperplasia induced by tamoxifen (64), which is a troublesome side effect of tamoxifen that may be a precursor to uterine malignancy (75). Thus AFPep might advantageously be used in combination with tamoxifen to obviate tamoxifen's toxicity. Secondly, as discussed above, it is reasonable to expect that AFPep may not engender acquired resistance because it uses natural signaling pathways and will not be perceived as being foreign to the body because it closely mimics the native human sequence of AFP. Since the usual animal models are of short duration and have not lent themselves to assessment of acquired resistance, we plan to use the ACI rat model of breast cancer which allows for extended treatment duration. These studies may well suggest that acquired resistance does not occur with AFPep.

AFPep has a Unique Mechanism of Action. AFPep is a multikinase inhibitor blocking the phosphorylation of c-Kit (Y703), FAK (S910), and MEK (S298, T386) and subsequently the phosphorylation of ERα (S118). Phosphorylation of S118 in ERα is needed for the transcription of growth signals in response to liganded ER (76). AFPep is different from tamoxifen in that it does not block binding of estrogen to ER (65). Instead, by blocking phosphorylation of ER it prevents formation of activated ER-E. In MCF-7 orthotopically xenografted in SCID mice and treated with AFPep, tumor biopsies indicated significant inhibition of phosphorylation of ERα, increases in ERβ, p21Cip1, Rb, and decreases in PCNA, suggesting that phospho-ERα inhibition is signaling a delay in the progression of tumor cells through the cell cycle, consistent with the significant

reduction in tumor growth rate seen between days 28 and 35 of this experiment (**Tables 2-3**). As future preclinical and clinical studies are carried out, these biomarkers will be very useful in monitoring biological activity of AFPep since they will change well before tumor burden.

Table 2. Effect of AFPep on Orthotopic Breast Cancer Xenograft Growth
AFPep initially inhibited, then stopped, growth of palpable tumors

Days after Tumor Implantation	Tumor Volume (mm^3)	
	Control	AFPep
21	156 ± 22	156 ± 22
22	Begin: Vehicle or	AFPep
28	487 ± 68	308 ± 30
35	897 ± 71	313 ± 45

MCF-7 human breast cancer cells (5×10^6) were injected into the mammary fat pad of SCID mice. Tumors were on average 6.7 mm in diameter (156 mm^3) on day 21 after implantation. Either vehicle or AFPep (100 µg/mouse) was given in 0.5 ml once daily for 14 days by oral gavage, beginning 22 days after tumor implantation. Tumors were harvested for biomarker analysis (see Table 4) on day 35 after tumor implantation. Mean ± SE of 3 replicate mice/group are shown.

Table 3. Effect of AFPep on Biomarkers in Breast Cancer

Biomarker	Change from Control, %
pERα	- 48 ± 5
pERβ	110 ± 15
Rb	38 ± 4
P21	82 ± 9
PCNA	- 53 ± 4

Tumor tissue was harvested for biomarker determination 35 days after tumor transplantation and biomarkers were assayed by Western blot. Change in band density in AFPep-treated mice relative to control group is reported. Mean ± SE from three replicate mice per group. These biomarkers can also be assessed by immunohistochemistry.

AFPep Prevents Breast Cancer. We have shown in prevention studies (64;68) that AFPep prevents the development of MNU-induced mammary cancers in rats (**Table 4**). Table 4 clearly indicates decreased incidence, multiplicity, and burden. The level of prevention was similar to that found with tamoxifen in similarly designed studies (64;68;77). It is impressive that AFPep (or any other agent) can prevent breast cancer in the face of a potent carcinogen such as MNU. Still, the model is quite artificial, using a bolus dose of a carcinogen not normally encountered by women and generating mammary cancers that are not similar to most that occur in women (78). Thus it is important to test AFPep against more realistic models of breast cancer such as that in the ACI rat model which is genetically primed to develop mammary cancer under the promotional influence of estrogen (78-86). Nevertheless, there is a very important proof of principle here: AFPep has the potential to be used to *prevent* as well as to *treat* breast cancer.

Table 4. AFPep prevents MNU-induced breast cancer							
Treatment	**AFPep Dose**		**Tumor Incidence**		**Multiplicity**	**Burden**	**Latency**
		µg/rat/day	%	p value	tumors/rat	cm^3	days
Saline	none	0	78	-	2.1	69	80 ± 31
AFPep	Sub-optimal	100	63	0.16	1.1*	39.1*	98 ± 33
AFPep	Optimal	270	40	0.02	0.5*	34.5*	88 ± 16
Incidence is defined as % of rats with one or more tumors at termination. Multiplicity is total number of tumors/number of rats in each group. Burden is the sum of the volume of tumors in each group. Latency is mean number of tumor-free days ± standard deviation. There were 30 rats in each group. * Statistically significant at $p < 0.05$ Data from (68).							

AFPep is Non-toxic. In studies to date no toxicity in mice and rats has been found with AFPep, even at doses as high as 1000 times the effective dose of 10 µg/mouse (66;68). Figures 6, 7, and 8 in reference (68) are typical of dozens of observations in which no side effects or toxicity has been seen when AFPep was administered to rats, mice, or dogs. Table 5 is a summary of the number of animals we have treated with AFPep in the past several years, none of which have exhibited side effects.

Treatment of pregnant rats with AFPep showed no disruption of the estrous cycle, no effect on fecundity or fertility, and no evidence of teratogencity. (87) While this bodes well for the potential of AFPep to serve as a breast cancer preventive agent, it also highlights the concepts of drug development that we discuss in this chapter. As noted in (87), the parent protein AFP disrupts reproductive tissues and function (14;15;27;88;89) but AFPep does not. This observation suggests that in isolating the anti-oncogenic site of AFP, the epitopes of that protein that adversely impact the reproductive system were effectively parsed out.

The favorable host toxicity profile cannot be over-emphasized since drug toxicity is one of the main reasons for failure of new drugs, and it is almost a requirement that an agent intended for use as a preventive be extremely well tolerated. We proffer that AFPep is efficacious without being toxic, which is true to date. The risk that there will be some side effects in humans being treated with AFPep for lengthy times is mitigated: by our extensive earlier studies in lower mammals; because AFPep is built on the human AFP sequence; because it is active at fetal physiological levels of AFP; and because the metabolites of AFPep are simple amino acids. At the very least, it is completely reasonable to expect that AFPep will be less toxic than cytotoxic agents such as doxorubicin and even SERMs such as tamoxifen, allowing use of AFPep as a therapeutic agent and probably as a preventive agent.

Table 5. Summary of AFPep treatment studies that showed no toxicity				
Species	**Treatment Duration** Days	**Autopsy Date** Days after Treatment	**Dose, Route** µg/animal/day	**Number of Animals**
Mouse	1	1	1 – 10,000 i.p. or p.o.	4000
Mouse	30	1	10 – 100 i.p. or p.o.	300
Rat	23	200	3 – 300 sc or p.o.	1000
Dog	1	None	10,000 i.v.	1
Mouse	5	1	2,000 i.v.	5
Mouse	1	5	10,000 i.v.	5
No toxicity was seen in the course of a variety of interventions, in three species. This lack of toxicity was not unexpected for four reasons: (1.) AFPep is derived from a natural human protein; (2.) AFPep has been designed/parsed/shaped to have only the anti-estrogenic, anti-breast cancer properties of AFP (59;61;62); (3.) The active, anti-breast cancer concentration of AFPep *in vivo* is well below that of the parent protein concentration in the blood of the human fetus (20); and (4.) The metabolites of AFPep are simple amino acids.				

Conclusions

We have described the development of AFPep, a new peptide derived from AFP that has enormous potential as a well-tolerated, efficacious drug for the treatment and prevention of breast cancer. We see no reason why some of the other functions (such as immunosuppression) associated with AFP (or other macromolecules) could not be isolated and developed into therapeutic agents using an approach similar to that outlined here. The homobiotic nature of AFPep approaches the Holy Grail of drug development, namely efficacy without toxicity. Efficacy as well as longevity of the biological action of a homobiotic drug is presumably based on re-engaging the natural biochemical circuitry of the cell, rather than utilization of interdiction of pathways in unusual processes as most xenobiotic agents do. Lack of toxicity is based on AFPep's mimicry of its parent protein, as well as the fact that metabolites of AFPep are simple amino acids (unlike xenobiotic molecules which often get metabolized to become even more toxic).

Presumably, the anti-breast cancer activity of AFPep derives from its anti-estrogenic activity, although its exact mechanisms of action have not been completely delineated and, like many drugs, probably will not be until AFPep has experienced extensive clinical use. If the pre-clinical data continue to manifest in humans, AFPep may be useful for the prevention of breast cancer, as well as treatment, highlighting the innate potential of homobiotic molecules.

Although AFPep was developed with a narrow focus on breast cancer, the question always arises as to whether it would work for other cancers, and preliminary cell culture evidence suggests that AFPep should be tested against a variety of maladies. In the tumor arena, AFPep has inhibited growth of uterine leiomyoma, glioblastoma, prostate and thyroid cancers. Mechanistically, given its inhibition of c-kit, AFPep has potential against gastrointestinal stromal tumors. From and endocrinological perspective, it may be useful in situations that require the tamping down of estrogen effects without their complete eradication such as in the treatment of uterine fibroids.

It seems likely that AFPep will have multiple future uses including treatment and prevention of breast cancer, due to its exquisite efficacy and tolerability trough it re-engagement of natural growth regulatory circuits and will become a significant addition to the pharmacopeia as well as serve as an example for future development of other homobiotic agents.

Figures.

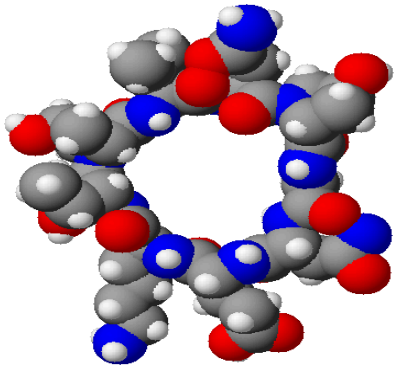

Figure 1. AFPep is *cyclo(EKTOVNOGN)*, the active site of AFP. Its metabolites are simple amino acids. *E* is at the six o'clock position; *O* is hydroxyproline.

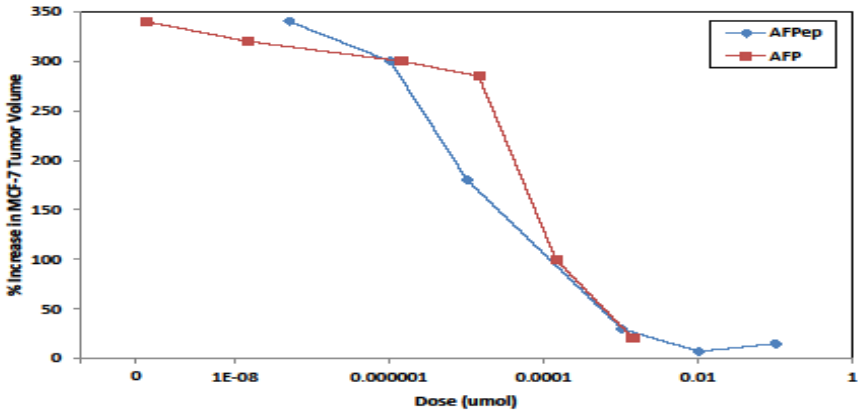

Figure 2. AFP and AFPep Inhibit Growth of MCF-7 Tumors. MCF7 tumor was implanted under the kidney capsule of SCID mice. Either AFP or AFPep were administered once daily i.p. for 30 days. Tumor volume was then measured and reported here as percent increase in tumor volume compared to Day 1. Each point represents the mean of three replicate mice.

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